# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| 03-25-2006 | FINAL PERFORMANCE REPORT | 7/15/04-10/14/05 |

**4. TITLE AND SUBTITLE**
NanoBench: An Individually Addressable Nanotube Array

**5a. CONTRACT NUMBER**
FA9550-04-1-0432

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Nalin Kumar, Ph.D.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
UHV Technologies, Inc., 450 South Freeway, Ft Worth, TX 76104

**8. PERFORMING ORGANIZATION REPORT NUMBER**
NBFinal-1

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
USAF, AFRL
AF Office of Sci. Research
4015 Wilson Blvd Room 713
Arlington, VA 22203-1954

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution is unlimited.

AFRL-SR-AR-TR-06-0125

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

An innovative approach called NanoBench was investigated during this project. NanoBench chip consists of an array of electric conducting nanoelectrodes that can be inserted inside a cell to measure intra-cellular signaling pathways, both electrically and optically. Du this project, (i) NanoBench chips with arrays of metallic tips were fabricated, (ii) very sharp tungsten probes were fabricated, functionalized used to demonstrate intra-cellular probing of cells, and (iii) full range of experimental techniques have been developed to perform optical electrical measurements of intracellular signals. As a model system, a sharp tungsten probe was inserted into a lysosomes that was swollen v sucrose (a sucrosome). Control over probe movement was sufficient to drive the probe into a sucrosome, and withdraw the probe. U withdrawal, the sucrosome collapsed, indicating that the vesicle was punctured by the probe tip. In a second model system, a lysosome labe with the acidotropic dye LysoTracker Red was touched with a metal tip, which popped the lysosome and released the dye. Future work v concentrate on making sharper probes and coating them with biological molecules to sense analytes in cells.

**15. SUBJECT TERMS**
Intracellular, nanotubes, CNT, nanoelectrodes, NanoBench, Pathways

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | Nalin Kumar, Ph.D. |
| Uncl | Uncl | Uncl | Unlimited | 42 | 19b. TELEPHONE NUMBER *(include area code)* 817-820-1500 |

## ACKNOWLEGEMENTS

We would like to thank the Univeristy of Texas at Dallas and von Ehr Foundation for providing support for the Nikon TE2000-L100 cell probing station used during this project.

Additionally, we would like to thank Gareth Hughes, Mike Nolan and Rishi Gupta of Zyvex Corporation in providing support in the operation of the TE2000-L100 equipment.

## Table of Contents

## Executive Summary

There is an urgent need to determine the fundamental functions carried out by molecular machines inside biological cells at nanometer scale, however, the current technologies such as fluorescence are limited to a 100nm resolution. Atomic force microscopy (AFM), even with its nanometer resolution has not been used for monitoring of internal signals because of lack of nanoscale probe arrays with individual addressability. Electron microscopy (EM), the other sub-nanometer resolution analytical technology, has also not been used for nanoscale probing of cells due to damage to cells by the electron beam and inability to maintain cells in vacuum.

An innovative approach called NanoBench was investigated during this project. NanoBench chip consists of an array of electrically conducting nanoelectrodes that can be inserted inside a cell to measure intra-cellular signaling pathways, both electrically and optically. During this project, (i) NanoBench chips with arrays of metallic tips were fabricated, (ii) very sharp tungsten probes were fabricated, functionalized and used to demonstrate intra-cellular probing of cells, and (iii) full range of experimental techniques have been developed to perform optical and electrical measurements of intracellular signals.

As a model system, a sharp tungsten probe was inserted into a lysosomes that was swollen with sucrose (a sucrosome). Control over probe movement was sufficient to drive the probe into a sucrosome, and withdraw the probe. Upon withdrawal, the sucrosome collapsed, indicating that the vesicle was punctured by the probe tip. In a second model system, a lysosome labeled with the acidotropic dye LysoTracker Red was touched with a metal tip, which popped the lysosome and released the dye. Future work will concentrate on making sharper probes and coating them with biological molecules to sense analytes in cells.

## 1.0 Project Objectives

The overall goal of this project was to demonstrate the feasibility of electrically measuring intracellular pathways using carbon nanotubes (CNTs) or metallic nanoelectrodes.

The major technical objectives of this work were as following:

- To fabricate NanoBench probes and attach them to the microprobes of a Zyvex nanomanipulator for bio-chemistry experiments.
- To use modified NanoBench chip in air to test biological sensing hypotheses.
- To develop techniques for chemically functionalizing nanoelectrodes.
- To design, fabricate and install hardware to place NanoBench and cells in a vacuum system, and to acquire data/signals from the nanoprobes with an electron beam in a FEI dual beam FIB/STEM/S100 Nanolab system at University of Texas.
- To study and quantify the signals obtained from single probes and to determine the signal to noise ratio (SNR) of these signals.

This project consisted of six tasks: 3 engineering related tasks and 3 biochemistry related tasks as listed below:

**Engineering**

Task 1: Fabrication of NanoBench and Nanoelectrodes

Task 2: Modification of the SEM/S100 and other Equipment

Task 3: Testing of the NanoBench

**Biochemistry**

Task 4: Functionalization of CNT nanoelectrodes

Task 5: Investigation of CNT nanoelectrode performance *in vitro*

Task 6: Detection of an intracellular analyte

## 3.0 Background

In order to develop the ability to detect very low levels of a wide range of warfare agents, development of cell and multi-cellular tissue-based sensors is being pursued. Such sensors rapidly detect and predict physiological consequences of both known and unknown agents by measuring the agents' effects on cellular activities. Due to lack of a requirement for prior knowledge of the threat, these biosensors are particularly suitable for bio-warfare defense against unknown and engineered threats.[1] Such devices that respond to a wide range of agents and accurately predict physiological consequences of exposure require fundamental understanding of cellular processes. Cellular responses from multiple cell-types including neurons, endothelial cells, immune cells, and stem cells, can be combined to improve the sensitivity and specificity of the biosensors.

Therefore, in order to enhance the utility of these biosensors, there is an urgent need to understand the fundamental functions carried out by protein networks inside biological cells. Biochemical signals passed through the protein networks control cellular operation and function, including the response to toxins. Subcellular elucidation of protein network function requires probing and imaging at the nanometer scale. However, currently, there is no technique to measure them at the nanometer resolution scale. The optics based fluorescence techniques are limited to a resolution of 100 nm or worse. Atomic force microscopy (AFM), even with its nanometer resolution cannot be used for monitoring of intracellular signals because of lack of nanoscale probe arrays with individual addressability. Electron microscopy (EM), the other sub-nanometer resolution analytical technology, has also not been used for nanoscale probing of cells due to damage to cells by the electron beam and inability to maintain cells in vacuum. Hence there is a need to develop new techniques that can determine the inside workings of a cell at nanometer resolution, however, currently, there is no technique to measure them at the nanometer resolution scale.

To address the above-mentioned opportunity, we proposed a novel carbon nanotube (CNT) based array measurment platform called NanoBench that will allow probing of live cells in real time with nanoscale resolution.

### 3.1 Current Techniques

Optical microscope has been the instrument of choice for studies of live cell behavior. With the development of scanning confocal microscopy, fluorescent protein tags and quantum dots, it is now possible to study distribution of molecules inside the cells.[2,3] However, these techniques have very poor resolution as compared to atomic force microscopy (AFM) or electron microscopy (EM). The best resolution obtained in the optical techniques is on the order of 100 nm.

For observation of biomolecules inside the cell, the resolution provided by optical techniques is not sufficient. In addition, the small number of molecules present inside a single cell for majority of proteins of interest is not easily detectable with optical methods. Most of the data obtained with light microscope has focused on highly expressed proteins such as actin

[1] JJ Pancrazio, et al. (1999) Development and application of cell-based biosensors. Annals of Biomedical Engineering. 27, 697-711.

[2] Lippincott-Schwartz, J. and Patterson, G.H. (2003) Development and use of fluorescent protein markers in living cells. Science, 300, 87-91.

[3] Stephens, D.J. and Allan, V.J. (2003) Light microscopy techniques for live cells imaging. Science, 300, 82-86.

and tubulin, or over-expressed reporter proteins. In order to address the resolution and sensitivity limitations of optical microscopy, electron microscopy and AFM have been used for studying small number of biomolecules.

Over the last 50 years, electron microscopy (EM) has played a crucial role in our current knowledge about the structure of biological cells and tissues. Initially, the EM was considered a low resolution tool useful for determination of morphology and pathology of cells and tissues until about a decade ago. The cryo-electron microscopy (ET) was a major step toward visualization of cells with a few nm resolution. However, in spite of this excellent resolution, the ET requires frozen samples (to avoid the vaporization of water from the samples inside the microscope), thereby precluding imaging of live biological samples. **This makes it impossible to measure sub-cellular signals inside a living cell with the current and projected electron microscope techniques.**

Scanning probe microscopy (SPM), commonly referred to as Atomic Force Microscopy (AFM), commonly used for imaging of atoms and molecules, has been applied to the study of cells. In AFM, a sharp conducting tip is scanned over the sample attached to a substrate. AFM has been recently utilized for many purposes in the field of biology and biotechnology as reviewed in many articles.[4] There are three main uses of AFM as listed below:

1. Molecular Imaging: In the contact mode, AFM tips have been used to obtain topographical images of biological molecules such as proteins, nucleic acids, and assemblies of these macromolecules.[5]
2. Functional Imaging: In this application, AFM tips are modified with specific functional groups to generate signal contrast resulting from certain chemical properties of the sample being examined.
3. Molecular Manipulation and Chemical Synthesis: Guthold et al have used the AFM tip as a nano-manipulator to manipulate biological molecules such as fibrin fibers and tobacco mosaic virus.[6] In addition they successfully used the carbon nanotubes as the AFM tips for imaging as well as molecular manipulation. Similarly, Muller et al have used AFM tips for chemical synthesis of nanostructures.[7]

The above listing is just an example of how the AFM has become the most popular tool for nanoscale probing of the biological molecules in a very short time. The level of information in AFM images depends on the size and shape of the tips used for imaging. Recently, the resolution of AFM technique has been improved with the use of carbon nanotubes (CNTs) as the probe tip. Umemura et al have used a CNT tip in an AFM to successfully resolve 10-nm pitch of RecA-DNA complexes.[8] Furthermore, it has been proven by many groups that carbon nanotubes are, by far, the best AFM tips available currently.

---

[4] H.X. You and L. Yu, 'Atomic force microscopy imaging of living cells: progress, problems and prospects', *Methods in Cell Science* **21**: 1-17 (1999).

[5] Cai, L., H. Tabata and T. Kawai, "Probing electrical properties of oriented DNA by conducting atomic force microscopy", *Nanotechnology* **12**, 211-216 (2001).

[6] Guthold et. al, "Investigation and modification of molecular structures with nanoManipulator", *J. Mol. Graphics and model.*, **17**, 187-197 (1999).

[7] Muller et al, "A strategy for the chemical synthesis of nanostructures", *Science* **268**, 272-273 (1995).

[8] Umemura et. al, "Atomic force microscopy of RecA-DNA complexes using a carbon nanotube tip", *BioChem. and BioPys. Res. Commun.* **281**, 390-395 (2001).

There is no doubt that AFM equipped with nanotube tips is a very important technology for nanoscale imaging and have contributed significantly to of our understanding in the structure of various molecules. However, AFM technology has several limitations as listed below:

1. Only One Tip: AFM typically does not have more than one tip, thus multiple points on a molecule cannot be monitored at the same time. It is possible to fabricate two or more tips side by side (shown in this application), but the issue of getting independent electrical signals from each tip is beyond the capability of state-of-the-art micro-fabrication technology. Currently employed fabrication techniques lack the resolution to attach wires to electrodes at the nanoscale. The state-of-the-art fabrication technology is limited to lines on the order of 100-200 nm, and the routing is extremely difficult when the number of electrodes goes beyond 100 or so. The limitation of one tip cannot allow the simultaneous probing of multiple spots; or **more interestingly, measurement of effects of applying one stimulus (electrical or otherwise) to one part of a molecule on other parts of the molecule in real time.** It is conceivable that the future development of techniques described in this application will one day enable AFM instruments to have multiple tips with excellent spatial, temporal and electrical resolution.
2. Limited Electrical Properties: The current AFMs measure surface morphology and chemical nature indirectly by measuring forces between the scanning tip and the surface. The scanning tunneling microscopy (STM) may be used but it does not work on non-metallic samples such as cells. Furthermore, the scanning nature of the SPM techniques requires scanning which is not possible if the SPM tip is embedded in the sample. **The intracellular pathways signaling signals are extremely small (few ions changing places) and the current techniques do not have the necessary signal to noise ratio (SNR) to measure these** due to the capacitance of the probe and attaching wires. It is believed that on-board amplification of very small signals will be necessary.
3. Difficult to Perform Studies of Time Dependent Phenomena: AFM is a spatial imaging technique and it is very difficult to perform studies of time dependent phenomena in real time. It is possible to scan back to the same location time after time but is not ideal with the AFM to do such studies. In such studies, the scan rate is no better than 10-60 seconds per image, hardly useful for real time monitoring of intracellular signals.
4. Other Issues: The AFM tips are very fragile and must be handled with a lot of care, resulting in significant down time and slow scanning speeds. Furthermore, the scanning of the AFM tip can cause deformation and damage to the sample due to a fairly high pressure within the contact area between the tip and the sample.

### 3.2 NanoBench Approach

To solve the above problems, we proposed a novel carbon nanotube (CNT) based array sample holder called NanoBench that will allow probing of single molecules and cells in real time with nanoscale resolution inside an electron microscope. The NanoBench substrate is essentially a two dimensional array of CNT nanoelectrodes fabricated on a micro-machined silicon substrate using a MEMS fabrication scheme. A small length of each nanotube sticks out of the one side of the insulating silicon dioxide layer such that the molecules/cells can be placed on it. Depending on the use, the molecules will be placed in between adjacent CNTs or on top of the CNTs (or CNTs are embedded into the cell/tissue for measuring the sub-cellular signals). Since the CNTs are very small (1-10nm diameter) and the distance between adjacent CNTs is only 10-100nm, the active area for a 100 x 100 CNT array is less than 10 um x 10 um.

In practice, the NanoBench substrate fabricated during this project will have a size of approximately 10 mm x 10 mm silicon chip with an active area of 1mm x 1 mm and will contain many (typically 100) 10 x 10 arrays, as described later. The thickness of the active area will be on the order of a few micrometers thick. The silicon substrate (0.5-1.0 mm thick) provides the rigid support and strength for the NanoBench substrate. The thickness of the oxide layer, the active area of the NanoBench substrate and the silicon support substrates are designed such that vacuum will be maintained on one side of the NanoBench substrate while exposing the other side to atmospheric pressure (Figure 1).

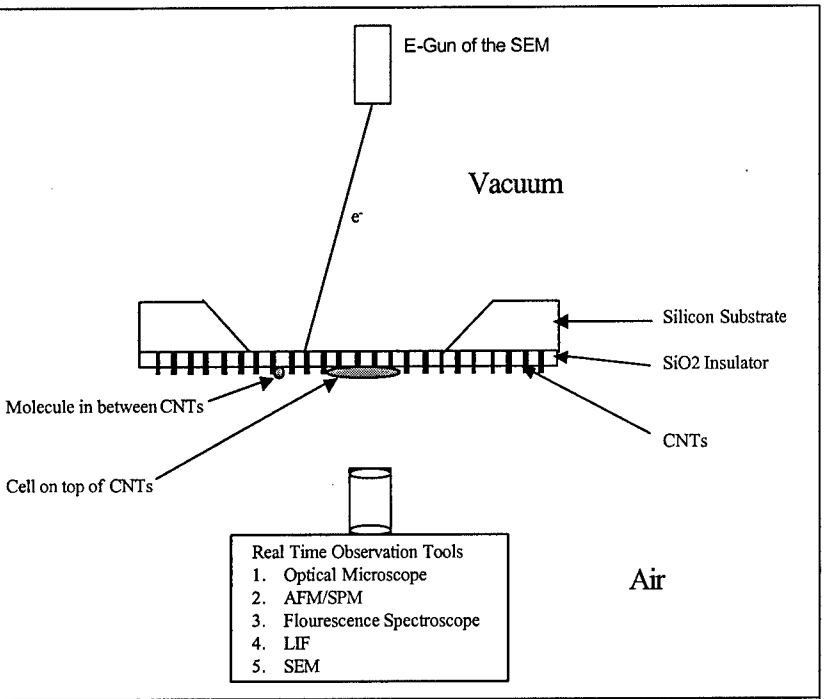



**Figure 3.1**: A conceptual diagram of the NanoBench for real time measurement of signaling pathways in cells.

The focused electron beam of the electron microscope (SEM/TEM) will be used to apply voltage to each CNT nanoelectrode. An electron beam is chosen because focused e-beams form the smallest probe size. In the FEI Nova 200 NanoLab system chosen for this project, it is possible to achieve e-beam spot size as small as 1 nm. This electron beam will be used to charge various nanoelectrodes on the NanoBench as described later. The same electron beam will also be used to determine (read) the voltage on the electrodes by measuring the energy of the secondary electrons emitted from the electrode (by measuring the energy of emitted electrons that is reduced by the electrode potential). If desired, both these actions can be accomplished at the same time, i.e. while the e-beam is charging a nanoelectrode, the emitted secondary electrons can be energy analyzed to obtain a real time feedback on the actual voltage of the nanoelectrode.

Various configurations of the nanoelectrodes that may be used for imaging, sample manipulation or probing are shown in Figure 2, and are discussed later in this proposal. In addition, the NanoBench substrate may be mounted in the electron microscope in several different embodiments based n the application and the microscope configuration. A few of these configurations are also discussed later. The NanoBench sample holder allows the sample to be kept in air or biological medium while the e-beam hits the other side of the NanoBench. This allows the cells to be kept alive in a biological medium while they are being tested. **The key advantage of the NanoBench is that the e-beam never hits the sample.**

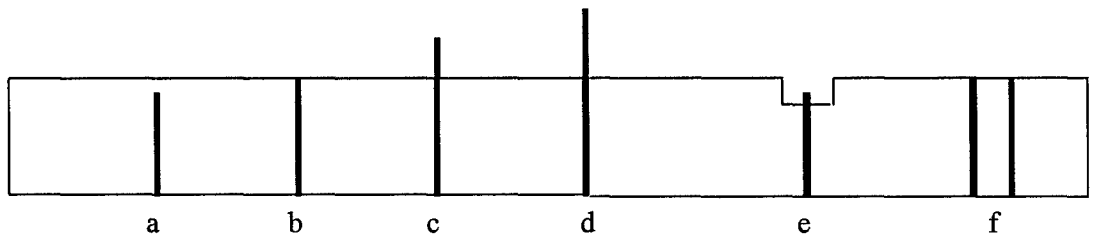



**Figure 3.2**: Various shapes of the CNT nanoelectrodes to be fabricated and studied: (a) embedded control and sensing Nanoelectrode, (b) surface contacting nanoelectrode, (c) & (d) penetrating nanoelectrodes, (e) sensing and imaging nanoelectrode, and (f) sensing and sizing nanoelectrodes.

### 3.2.1 Signal Sensing with Functionalized Carbon Nanotubes:

In this project, the sub-cellular signal sensing will be accomplished with CNT nanoprobes inserted into cells. Micro-needles and other small probes including silicon micro-machined needles have been used for probing various tissues for many decades.[9] It has been shown that these biosensors can easily measure electrical signals generated directly or indirectly by the biochemical reactions, however, due to their large size, the directly measured electrical signals correspond to an average of multiple cells/molecules. Various biochemical signals may be measured by coating the needles with various chemicals such that their interaction with other molecules generates electrical signals that can be measured. CNTs' unique electronic, chemical and mechanical properties make them very useful for biosensors and have attracted a lot of attention recently. In the last few years, studies have shown that CNTs can impart strong electrocatalytic activity to the electrochemical devices and also reduce the biofouling (which has been the reason for most biosensor degradation). CNT modified biosensors have been used to show improved electrochemical behavior for catecholamine neurotransmitters, cytochrome c, ascorbic acid, NADH, and hydrazine compounds. However, in all of the above cases, a large number of CNTs were used collectively to act as the probe (mainly because of isolating and accessing individual CNTs).

#### 3.1.2.2 Real Time Signal Measurement

The key innovation of the proposed project is the use of an electron beam to electrically access the nanometer sized 2-D CNT array (that otherwise can not be wired with traditional wiring techniques). The core electron beam testing technology has been developed for non-destructive high speed testing of silicon chips and has been described only briefly here intuitively, rather than rigorously that can be found in detail in a recent book.[10] The state-of-the-art e-beam testing equipment currently being used for high speed ship testing can have 10nm probe size with a 50 picosecond temporal resolution (20GHz frequency response).[11]

---

[9] Rutten, WLC, "Selective electrical interfaces with the nervous system". Ann Rev Biomed Eng., **4**. 407-452, 2002.

[10] Thong, J.T.L., Ed. "Electron Beam Testing Technology", Plenum Press, New York, 1993.

[11] Thong, J.T.L., Ed. "Electron Beam Testing Technology", Plenum Press, New York, 1993.

Signal monitoring with e-beams is hard to understand, but is easily accomplished with modern electronic instrumentation. It is based on the principle that the energy of the secondary electrons emitted from a charged electrode is reduced by the electrode potential as shown in Figure 3. If the secondary electrons are collected and their energy is analyzed, the voltage on the electrode can be determined. Typically, a simple mesh detector is used as the electron collector and the collected electrons are accelerated towards a PMT (photo multiplier tube), **thereby resulting in a very large gain and high signal to noise ratio**. This signal is analyzed with fast electronics to determine the minimum cut-off of the collected electrons, which represents the electrode voltage. Since all the changes in detector potential are done at a rapid rate in synchronization with the e-beam position and energy, the detected signal can measure the CNT nanoelectrode voltage in real time. We believe that we can obtain good signal to noise performance and high sensitivity for these signal measurements due to the very small size of the NanoBench holder.

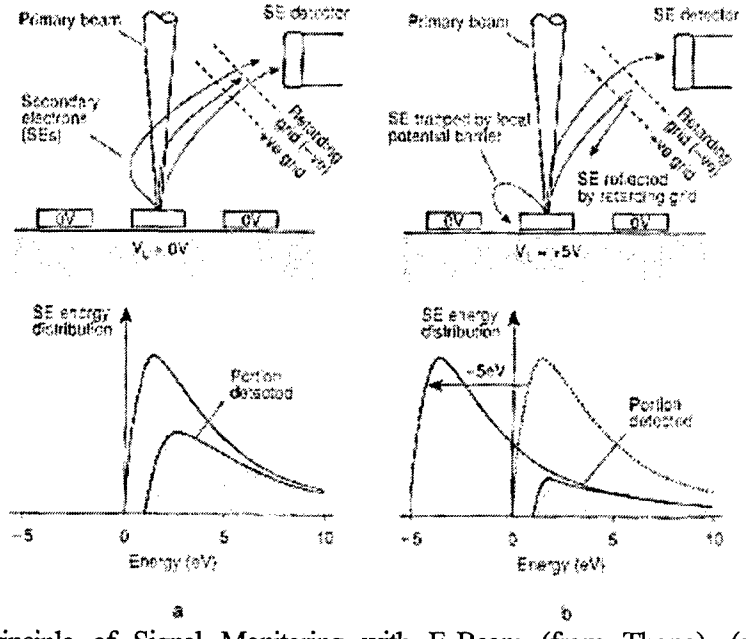



**Figure 3.3**: Principle of Signal Monitoring with E-Beam (from Thong). (a) Electrode being probed at 0 volts. A large portion of the secondary electrons (SE) have sufficient energy and pass through the retarding potential mesh detector. (b) +5V applied to the electrode. Lower energy SEs are trapped by the retarding field and the detected SE distribution is smaller.

*Waveform Measurement*: The combination of changing the voltage applied to a CNT (or applied between various CNTs, test and refernce nanoprobes) will allow application of a voltage to different parts of a cell, while the real time potential measurement on a third CNT will correlate the current measurement at this node. We believe that this approach will allow typical voltammetric and amperometric measurements with CNT nanoelectrodes, typically used for biological sample measurements.

*Signal to Noise Ratio (SNR) for Monitoring Signals with CNT Nanoprobes*: The key question in this project is whether SNR of the measurement is good enough to monitor intracellular signals. For most of the intracellular signaling, we have to measure extremely small signals when few ion/electrons reach/leave the CNT nanoprobe. As a simple calculation, the amount of

CNT potential change is determined by the capacitance of the electrode, the leakage of current through the insulator and the number of electronic charge change on the dectrode. Ignoring the charge leakage effect as a first approximation, the voltage rise of the electrode is given by the relation, V = ne/C, where n is the number of electronic charges, e is the electronic charge and C is the capacitance of the electrode. Assuming the capacitance to be 10E-15 farad for a free standing single CNT, the transient change in the CNT voltage will be approximately 160 microvolts per electronic charge. Thus, **change of 10 electronic charges will produce a transient voltage change of 1.6 mV**. This is a voltage that can be measured with proper design of the detector and photo-multiplier tube (PMT) based low noise amplifiers resulting good SNR. The key factor that helps in improving the SNR is that we are NOT counting the electronic charges (which is very small, giving poor SNR), but we are measuring the change in the energy (distribution) of the emitted electrons with respect to steady state condition. This indirect measurement is the key to obtaining very good SNR.

Furthermore, in practice, the SNR may be further improved by fabricating control electrodes on the NanoBench such that there is always several reference nanoelectrodes that are probed at the same time and used to determine the steady state background signal. The EM e-beam energy (typically on the order of 500-1000 eV for most materials) will be adjusted such that the number of secondary electrons is equal to the number of primary e-beam electrons (per unit time). Thus, there will be no change in voltage on the reference nanoelectrodes, and the secondary electron energy distribution is accurately known. However, as the charges due to intracellular signals reach the CNT nanoelectrode, the emitted electron energy distribution will change and can be very accurately determined by scanning (applying) the mesh detector voltage very accurately. **Again, in this measurement, even the PMT noise is not a factor** and the gain can be increased more than that used typically in scanning electron microscopes (where the detector electronics counts the number of electrons).

Another method of monitoring the intracellular pathways is to measure the impedance (or conductivity) between two adjacent CNT nanoelectrodes with the electron beam. This impedance will change when the nanoelectrodes are activated due to intracellular signals.

### 3.1.3 Using NanoBench for Real-Time Intracellular Pathway Monitoring

Figure 3 shows a schematic of feasibility experiments to be performed in Phase I of this SBIR project. While methods for NanoBench fabrication are being developed, biochemical experiments with a single nanotube will be begun. The objective will be to determine the sensitivity of the nanotube electrode (nanoprobe).

For the feasibility demonstration, we will use MAPK (MAP kinase) pathway, which is a central pathway in cellular function and survival.[12] The three pathways regulated by ERK (extracellular singal-regulated kinases), JNK (c-jun amino-terminal kinases) and p38 regulate a variety of physiological processes. The activity of the protein kinases involved in the MAPK pathways are controlled by addition and deletion of phosphate groups to the proteins. The addition of the phosphate is catalyzed by an upstream kinase protein and the deletion is

[12] Johnson, G.L., and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science, 298:1911-1912.

catalyzed by a MAPK phosphatase. Phosphate groups are negatively charged groups and phosphate addition to a protein significantly changes the electrostatic characteristics of the protein, including its interaction with other proteins, its catalytic ability and subcellular localization.

We believe that the CNT nanoelectrodes will be able to differentiate between phosphorylated MAPK and unphosphorylated MAPK due to differences in the charge characteristics. We will test this hypothesis by making two identical nanoelectrodes, coating both with an antibody that captures both unphosphorylated and phosphorylated protein, and allowing one to react to unphosphorylated MAPK and the other to phosphorylated MAPK *in vitro*. Thereafter, we will perform cyclic voltammetry using e-beam charging and reading as described earlier.

In addition, the CNT nanoelectrodes might also be able to detect the actual process of phosphate group in addition to a protein. In this case, a MAPK protein bound to a nanoelectrode will be phosphorylated by the upstream kinase and the addition of phosphate to MAPK will change the electrical characteristics of the electrode. We will test this scenario by fabricating a nanoelectrode and coating it with unphosphorylated MAPK protein. MAPK protein will either be adsorbed on the nanotube or covalently linked using thio-ester linkage as described earlier (Pierce Endogen). For *in vitro* testing, the nanoelectrode carrying the MAPK proteins will be exposed to a solution of upstream kinase. The change in potential will be measured using e-beam reading. Similarly, the effect of removing phosphate groups from a fully phosphorylated MAPK protein will be measured using a MAPK phosphatase.

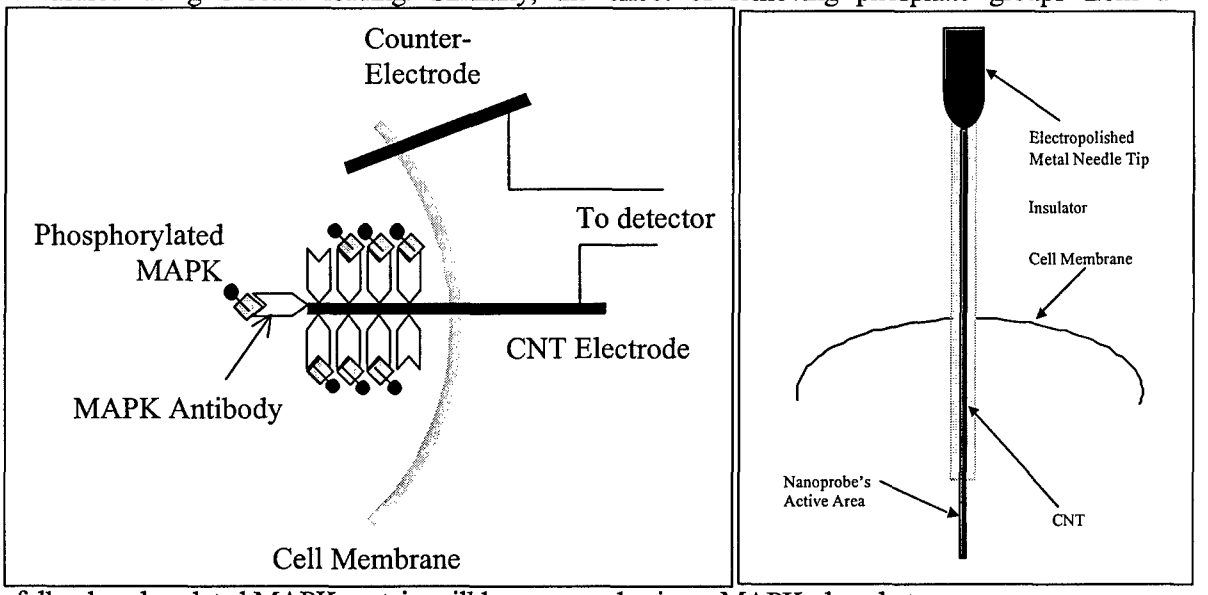



**Figure 3.4:** (a) A schematic of the single CNT nanoprobe measurements to be performed in Phase I. A CNT nanoelectrode and a counter electrode coated with anti-MAPK antibodies will be mounted on a nanomanipulator at UT, Dallas and carefully inserted into a HeLa cell. (b) Details of a single CNT nanoelectrode showing various conducting and insulating regions to control the active area of the nanoprobe.

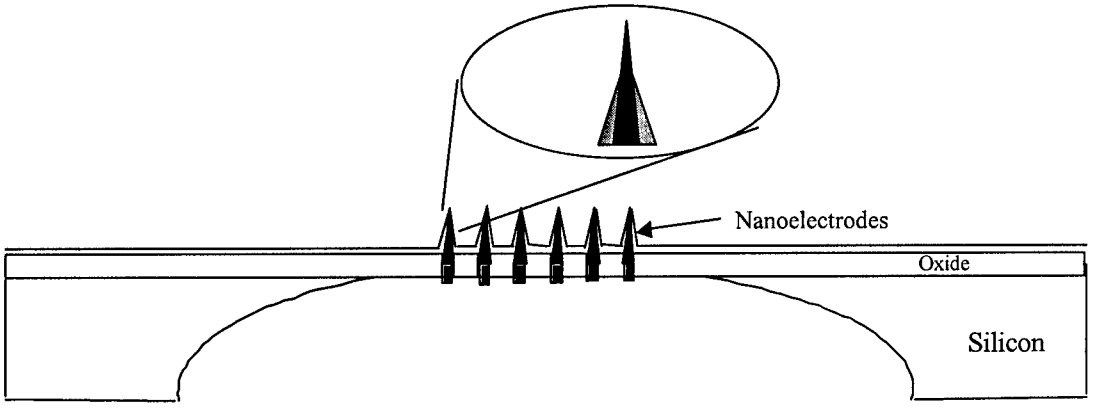
# 4.0 Experimental Details and Results

## 4.1 Task 1: Fabrication of NanoBench and Nanoelectrodes

During this project, several chips containing nanoelectrodes and nanoelectrode arrays (NanoBench) were designed and fabricated. In addition, we developed technologies to produce extremely sharp metal electrodes that could be used with the Zyvex probe station. The experimental details are given in the following sections for these chips and tungsten needles.

*4.1.1 **Fabrication of Through-Hole NanoBench Chips**:* A NanoBench chip containing through-hole contacts is schematically shown in Figure 4.1. It shows a silicon/quartz chip with sharp electrodes on one side of the chip and electrical connections on the other side of the chip. The electrical connections pads are connected to the nanoelectrodes with metallized via holes. The shanks of the nanoelectrodes and chip surface is coated with high quality insulator to minimize conductance between adjacent electrodes. Only the tip of the electrodes is exposed so as to obtain very high shunt impedance when the nanoelectrodes are inserted inside the cells.

**Figure 4.1**: A schematic diagram of a NanoBench Chip




The fabrication process is schematically shown in Figure 4.2. The process involves thinning a silicon or quartz wafer by mechanical and chemical polishing to a thickness of a few micrometers. These thinned chips were mounted inside the FEI NanoLab200 focused ion beam system (shown in Figure 4.3) at UTD and the gallium focused ion beam was used to etch holes at the desired locations as shown in Figure 4.4(i). The focused ion beam deposition of platinum was used to fabricate nanoelectrodes on the front side of the chip as shown in Figure 4.4(ii). Similarly, the holes were filled with platinum from the other side as shown in Figure 4.4(iii).

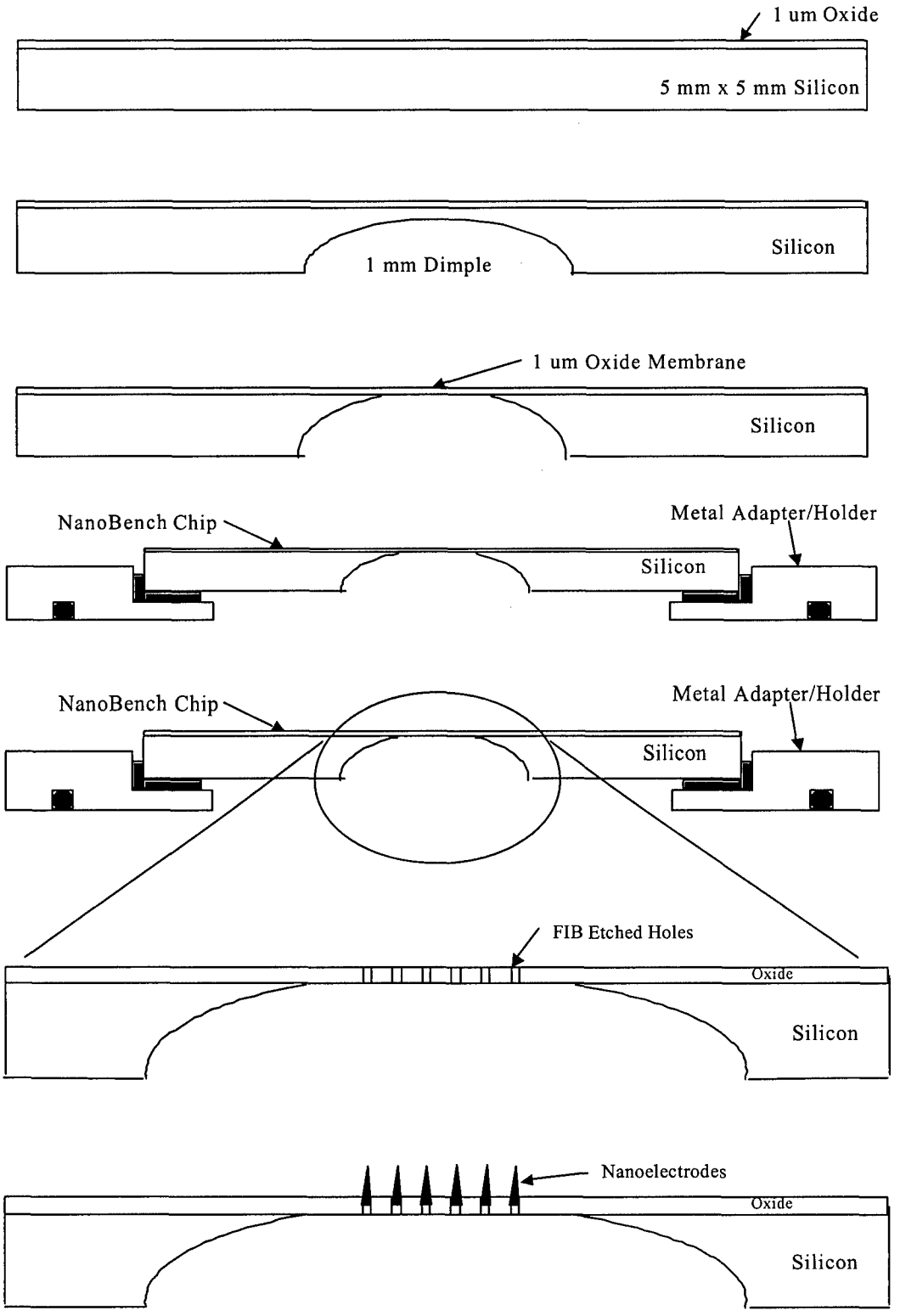



**Figure 4.2**: A schematic diagram of NanoBench Chip fabrication process

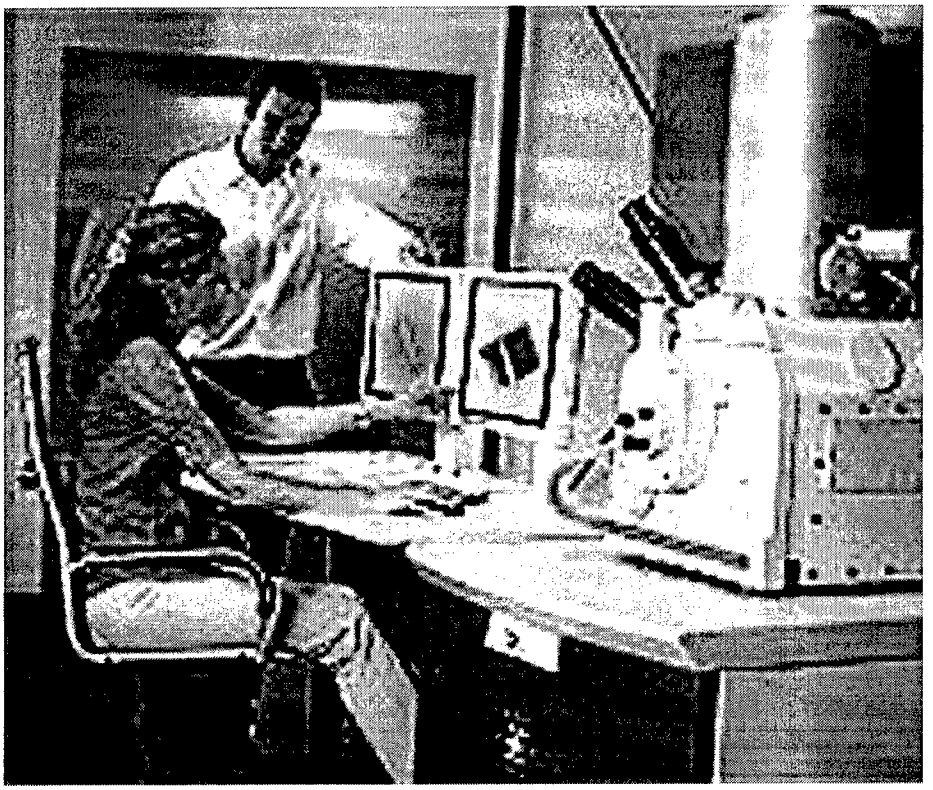


**Figure 4.3**: A photograph of the FEI Nova 200 Focused Ion Beam system used in the NanoBench Chip fabrication process

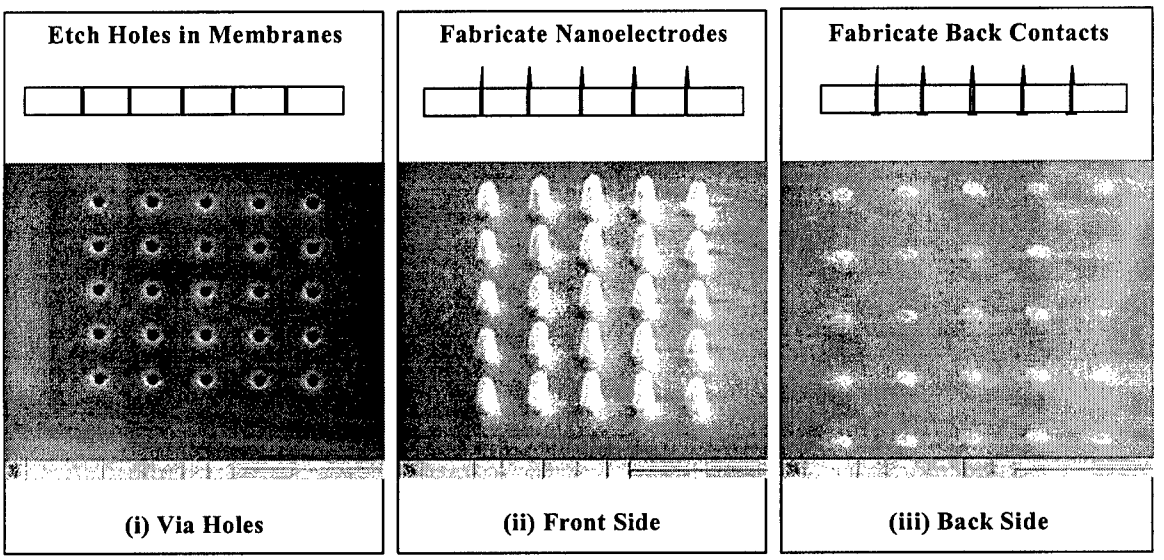



**Figure 4.4**: SEM micrographs of a 5 x 5 nanoelectrode array with 1000 nm inter-electrode distance and 100 nm nanoelectrode tip radius The size bar in each case is 2 microns long.

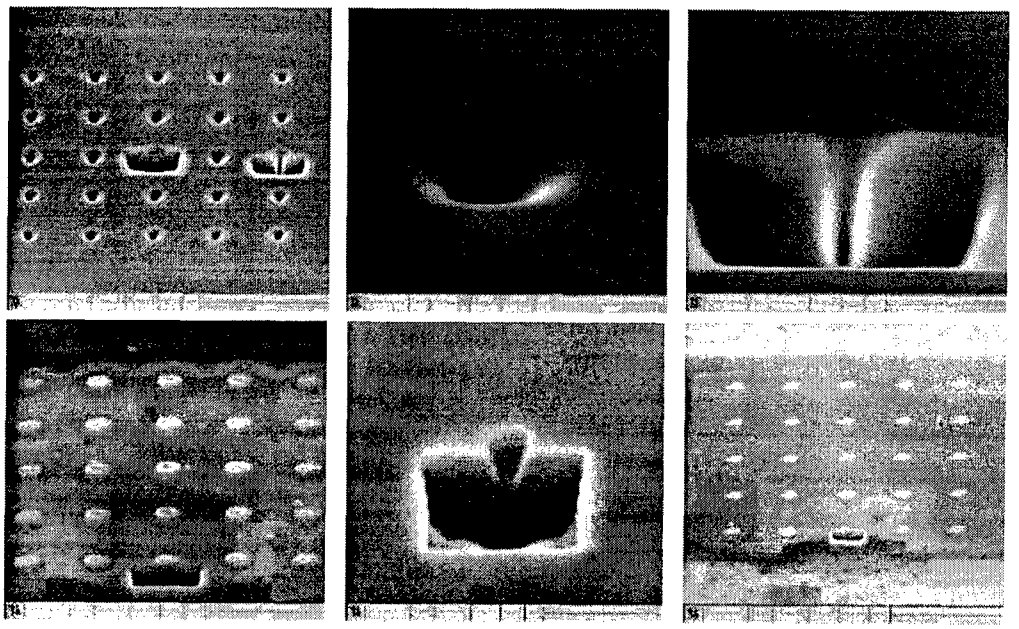


**Figure 4.5**: SEM micrographs showing various details of a 5 x 5 nanoelectrode array

These nanoelectrodes have a tip radius on the order of 100nm. Some of the details of these nano-machined features are shown in Figure 4.5. In addition, we devised techniques to fabricate sharper (20nm) nanoelectrodes in an array size as large as 20 x 20 arrays with interlectrode size as small as 200nm as shown in Figure 4.6.

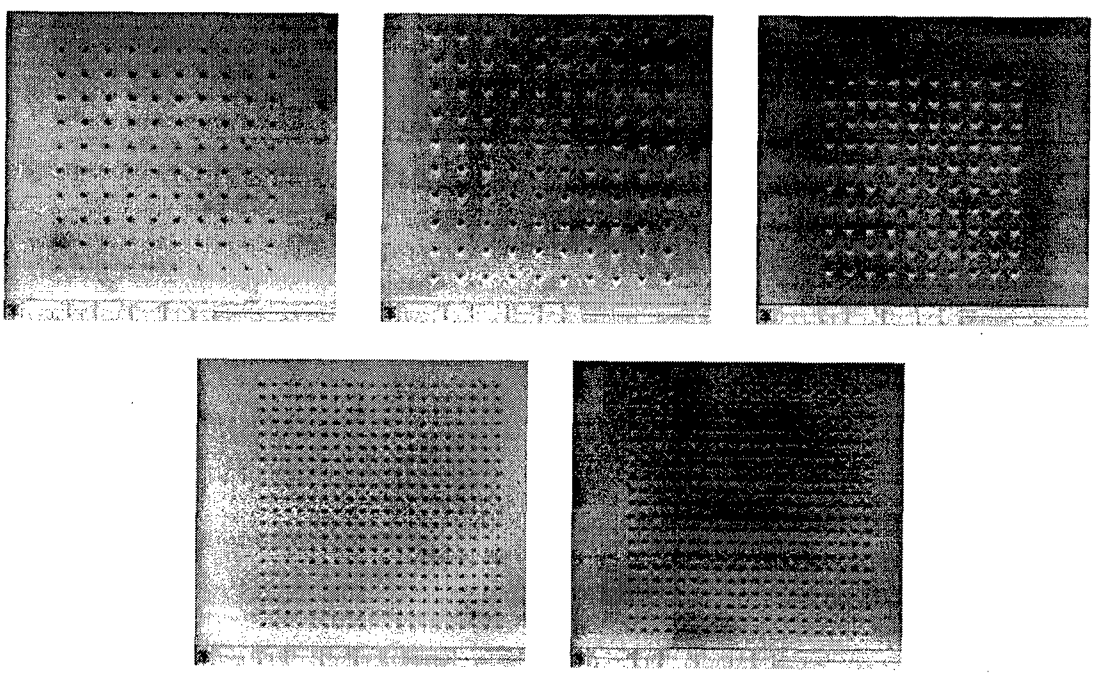


**Figure 4.6:** SEM micrographs of hole arrays for 10 x 10 and 20 x 20 nanoelectrode arrays

*4.1.2 **Fabrication of Planar NanoBench Chips**:* NanoBench chips described above are ideal when used for nanoscale testing in a scanning electron microscope. However, during the course of this project, it was realized that we needed confirmation of results obtained by e-beam measurements. Thus, it was decided to fabricated modified NanoBench chips (called Planar NanoBench chips) for testing in air by directly attaching the nanoelectrodes to electrical wiring.

Figure 4.7 shows photographs of a 18mm x 18mm glass chip that was designed for inserting nanoelectrodes in cells under an optical microscope. This design included 20 contact pads (16 for signals and 4 for ground) to connect the chip to a printed circuit board

*** 18mm x 18mm glass chip**

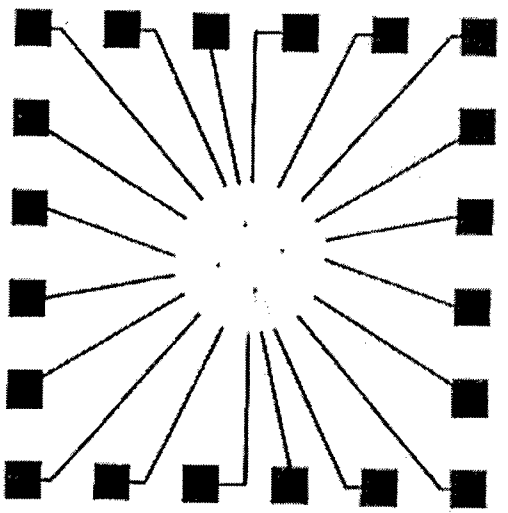
*** 0.5mm thick glass for use with Inverted microscope**

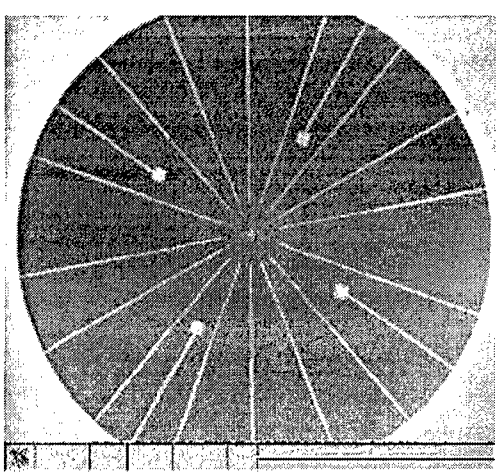
*** 16 electrodes (1.5 um lines)**

*** 4 Ground pads (10 um)**

*** 1mm contact pads**

*** Mounts to PCB**





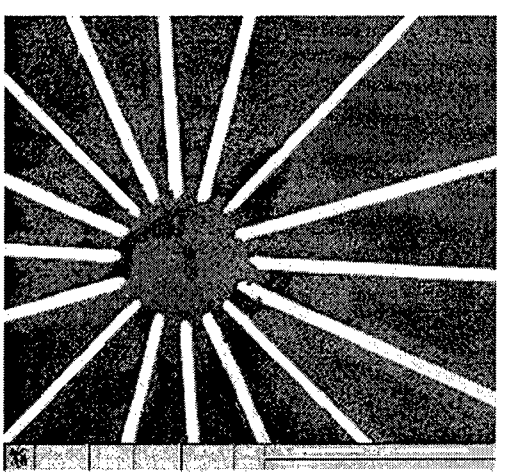


**Figure 4.7**: General arrangement of the 16 electrode Planar NanoBench chip fabricated by standard photolithography process on 4 inch glass wafers.

(PCB) and to the electrophysiological signal measurement instrumentation. These pads led to the center of the chip with metal lines of decreasing width, terminating in a 30 micron diameter circular arrangement. The pads and lines were made by standard photo-lithography process using 4 inch wafer processing. After metal patterning, the individual chips (18mm x 18mm) were diced and made ready for nanoelectrode fabrication in the UTD FIB system.

In the first process, high quality parylene coating was vapor deposited on center of the chip to insulate the metal lines on the surface. Photolithography was again used to remove Parylene from the center 50 micron diameter section so as to expose the ends of the 16 electrode connection lines (and also 4 ground pads). At this point the chip was placed in the FIB system and the FIB enhanced platinum deposition was used to fabricate nanoelectrodes as shown in Figure 4.8.

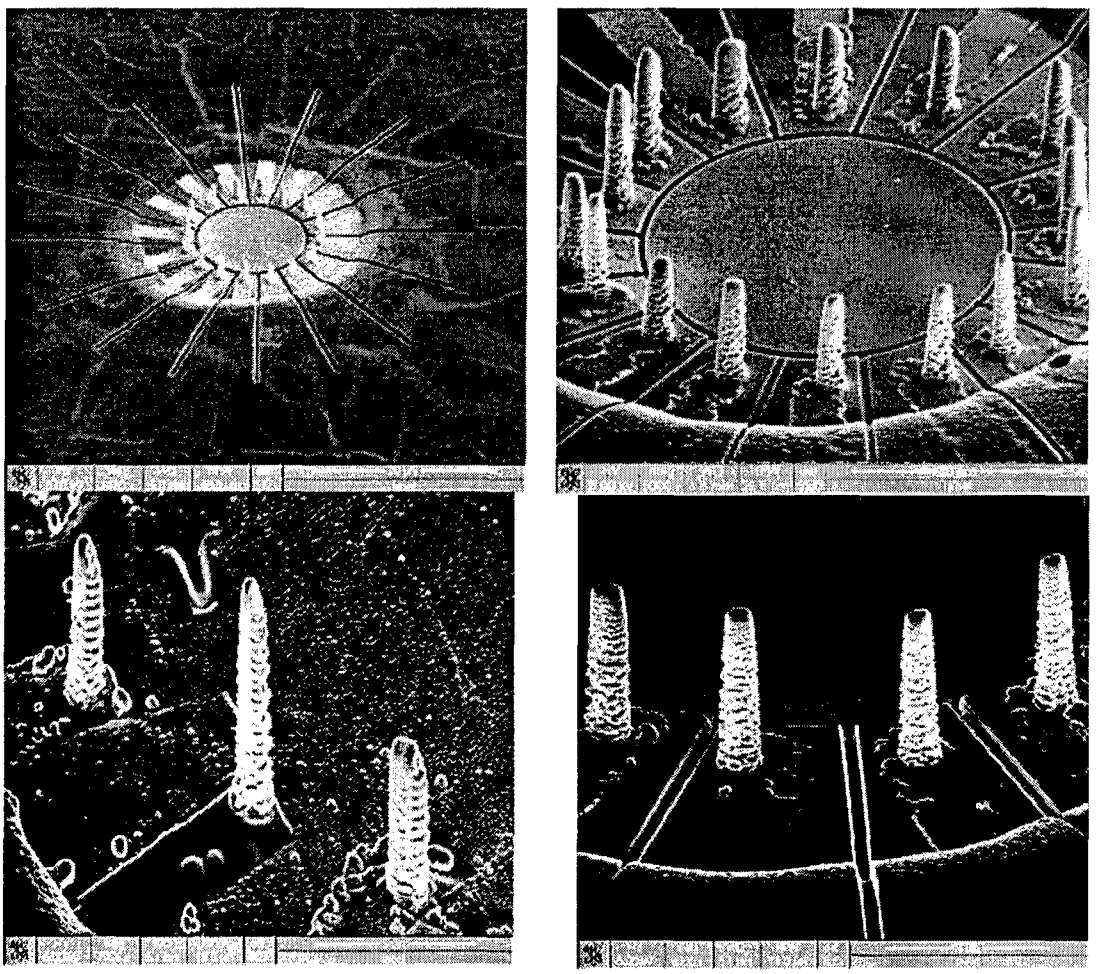


**Figure 4.8**: SEM micrographs the center section of the 16 electrode Planar NB Chip

One of the issues with the above design is that the nanoelectrode shanks are not coated with an insulator layer. To fabricate nanoelectrode chips with very high interelectrode insulation and high shunt resistivity, we fabricated NB chips using a modified process as described below.

In the second planar NB chip fabrication approach, platinum tips were formed on the glass chip before parylene deposition (4.9 a and b). After formation of the nanoelectrodes, the parylene was deposited on the whole chip surface covering the nanoelectrodes as well (c). At this point, FIB was used again to expose the tips of the nanoelectrodes (d). The results of this fabrication process are shown in Figure 4.9.

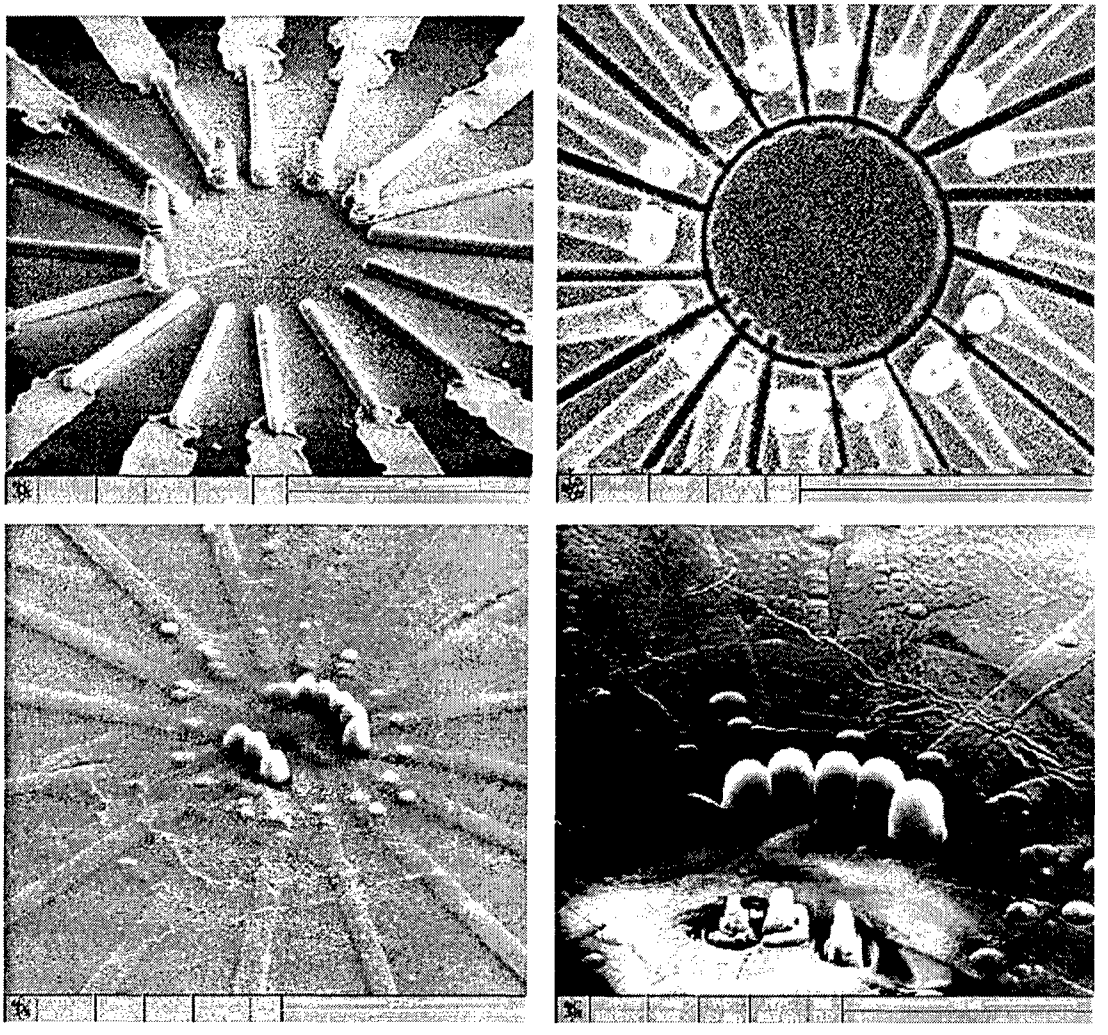


**Figure 4.9**: SEM micrographs of the center section of the 16 electrode Planar NB Chip at various stages during fabrication using the second process.

We were able to easily and consistently fabricate 5 micron tall nanoelectrodes. The tips of these nanoelectrodes could be sharpened by ion beam etching in the FIB system (e.g. by using a donut type etch pattern). These electrodes can have tip radii in the 50-100nm range.

***4.1.3 Fabrication of Tungsten Nanoelectrodes***: In parallel with the NB chip development, we also developed fabrication techniques for making tungsten nanoelectrodes that could be used for:

**(i)** Probing of live cells on a Zyvex probe station to calibrate/validate the NanoBench measurements.

**(ii)** Probing of live cells on a Zyvex probe station to compare electrical data from intracellular experiments with fluorescent measurements, and

**(iii)** Use as the electrode for attaching carbon nanotubes for future CNT based intracellular probing.

In the beginning of this project, we purchased commercially available tungsten needles from several sources, and used them for cell probing experiments. What we found that electrodes from most of the vendors had a tip on the order of 1-3 micron (1,000-3,000 nm). Only Veeco made claims of tip size in the 250nm range. Recently, Zyvex is producing probe tips in the 100nm range, which were used in our initial experiments. However, we found that commercially available tungsten tip were highly inconsistent. Furthermore, it was necessary to make the shank of the needle insulating for our electrical measurements. As far as we can determine, these insulating shank electrodes are NOT commercially available. Thus, we were forced to develop out own technology to develop the tungsten nanoelectrodes for this project.

The initial tungsten nanoelectrodes were fabricated by a typical electrochemical etching process using the setup shown in Figure 4.10. In this setup, a tungsten wire (typically 250 microns diameter) is dipped into one mole concentration sodium hydroxide solution and a small dc/ac voltage is applied. For obtaining the long shank for the needle, the needle is pulled up at a controlled rate using a stepper motor. The voltage source can alternate between AC and DC regimes. Several Tungsten needles, as single or multiple needles, were etched to different tapered shapes and tip radiuses. A special care was taken as to ensure that the needles were immersed vertically into the etching solution. The etching process is a three-step procedure:

**Step 1:** This step is carried out to ensure surface oxide and contaminants removal.
- Insert needle(s) in the etchant to a depth of ½".
- 2 Volts AC for 10 seconds.
- 2 Volts DC for 10 seconds.
- 3 alternate switching from AC to DC for 2 seconds at each mode.
- Turn the power off and pull the needle(s) out of the solution.

**Step 2:** This step is carried out to taper the needle and give its tip a desired radius.

- Insert the needle(s) 3mm deep into the etchant.
- Etch under 2 Volts AC for 2minutes.
- Turn the power off and remove the needles.

**Step 3:** This step is the most critical to give the tip its final radius without adding any additional radii between the tip and the shank.

- The needle is immersed 50 microns deep into the solution at 2 Volts AC till all chemical reaction stops.
- Turn the power off and remove the needle(s).

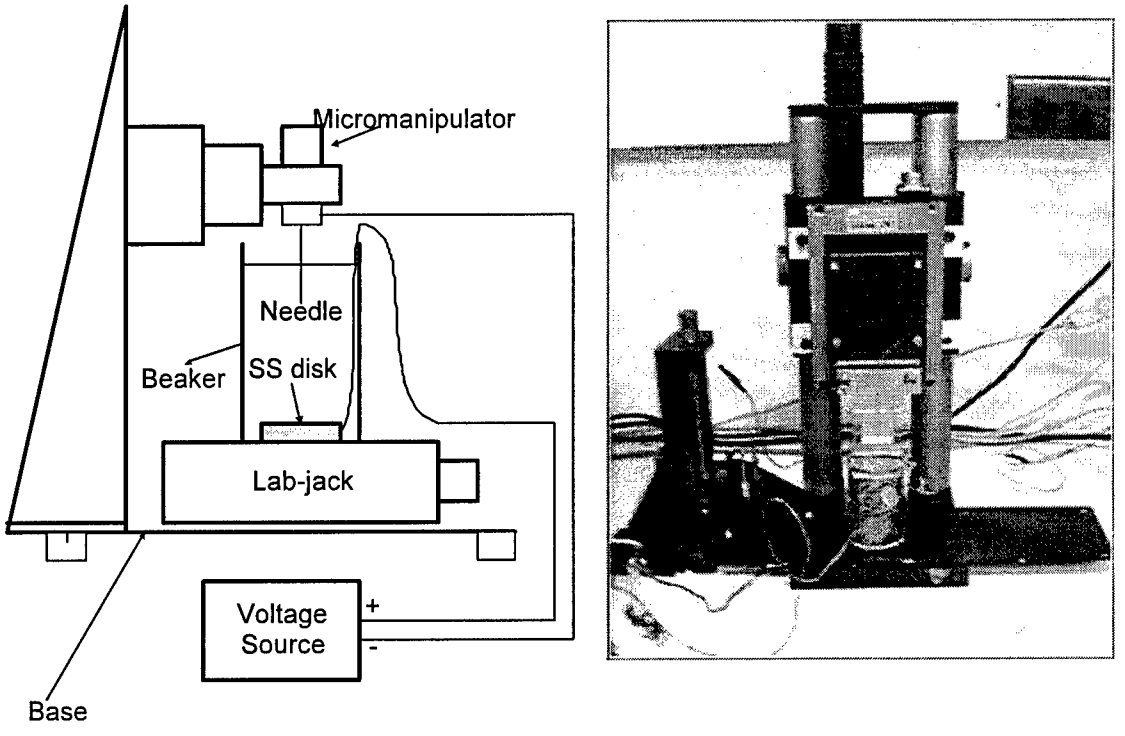



**Figure 4.10**: Schematic diagram and a photograph of the electrochemical etching setup used for tungsten needle fabrication

Using this system, several tungsten needles were fabricated under different conditions. Some of these are shown in Figure 4.11. However it was found that it was very difficult to consistently obtain tip size below 250nm with electrochemical polishing. Thus to obtain still finer tips, we used gallium ion beam milling of these electrochemically etched electrodes. As shown in Figure 4.12, the combined electrochemical etching and ion milling gave tip size below 100nm routinely.

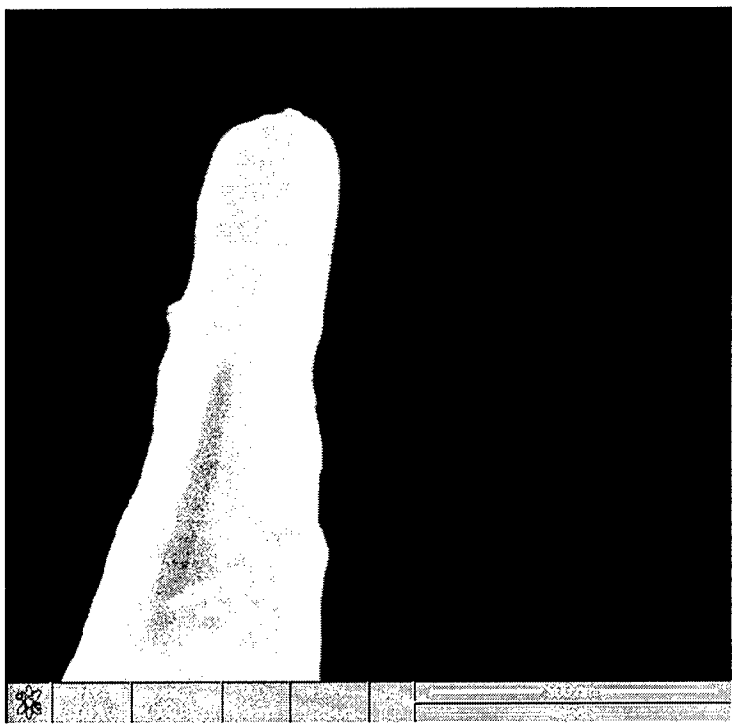


**Figure 4.11**: Photographs of the electrochemically etched tungsten electrodes (typically 200-300nm tip size).

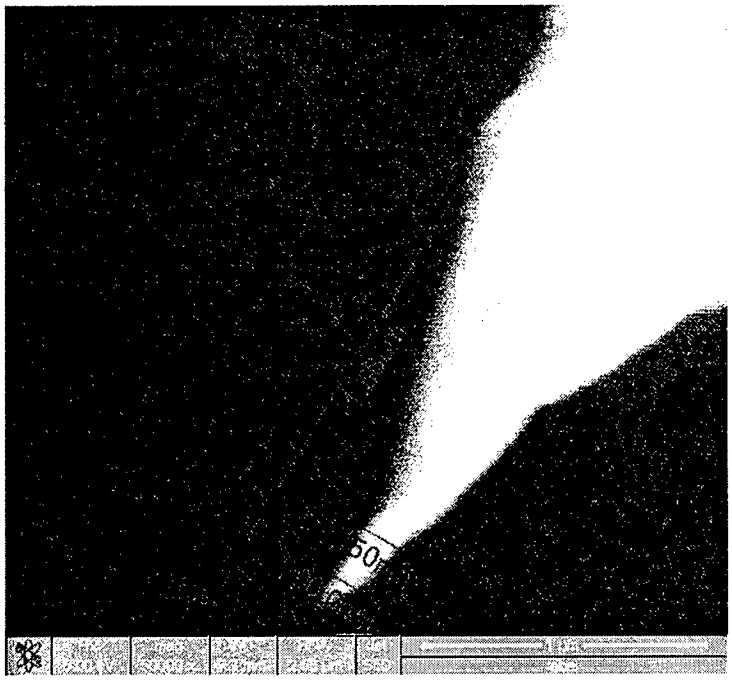


**Figure 4.12**: Photograph of an electrochemically etched tungsten electrodes followed by focused ion beam milling to obtain tip size below 100nm.

One of the problems in cell probing with metal electrodes is that the whole metal needle is immersed in the medium thereby the shunt resistance is very low, decreasing the measured signal level. Due to this, the metal electrode must be coated with an insulating layer except for the tip. During this project, tungsten electrodes were first coated with a thin layer of spin-on-glass (SOG) by dipping the electrodes in SOG and properly baking them. The SOG on the tip was removed by focused ion beam milling. Figure 4.13 shows SEM photos of two nanoelectrodes where the shank was insulated with SOG.

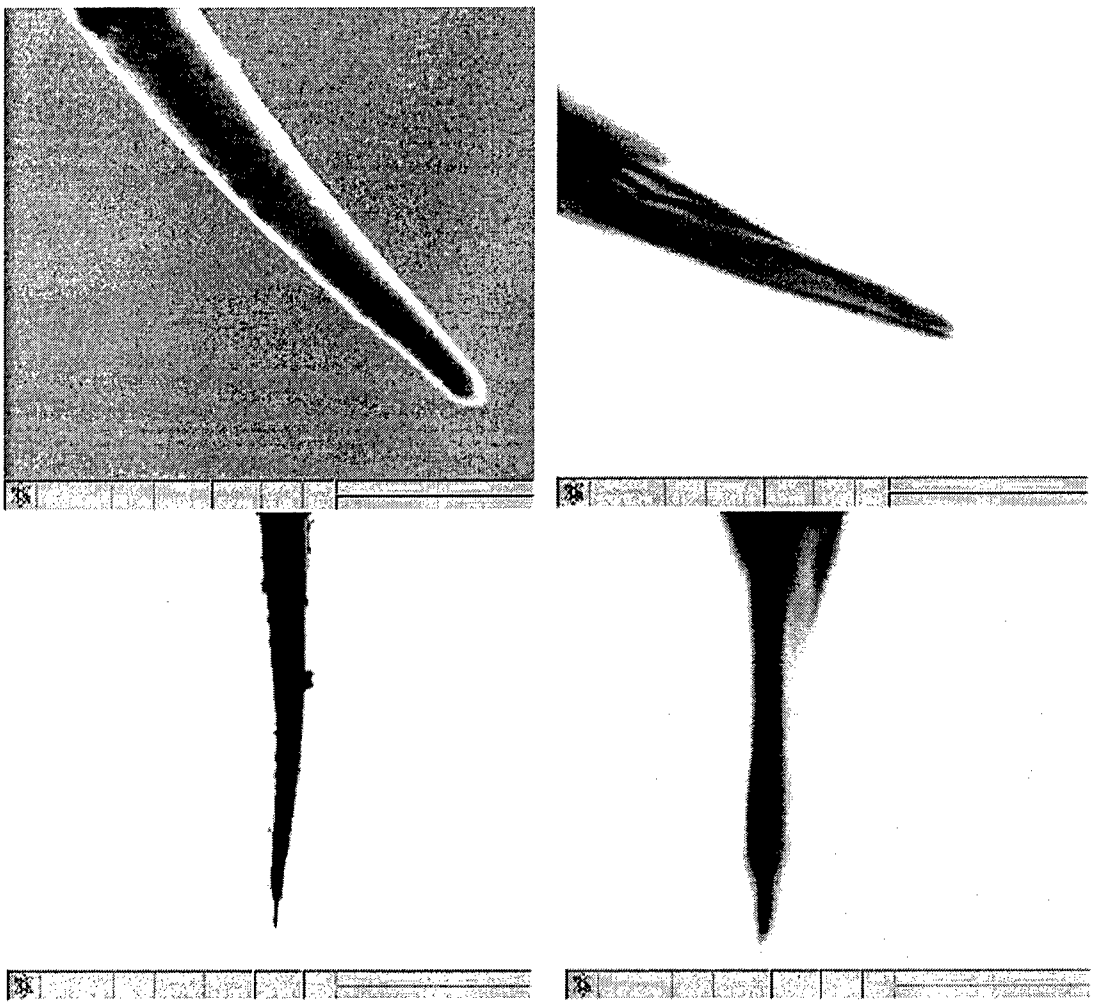


**Figure 4.13**: SEM Photographs of two SOG coated tungsten nanoelectrodes before and after removal of SOG from the tip

However, it was found that SOG is not a very good insulator when these electrodes were dipped in medium. Thus, we changed to the use of parylene as the insulator coating as described below.

Parylene is a vapor deposited polymer typically used for passivation of electronic circuits. Figure 4.14 shows SEM Photographs of a parylene coated tungsten nanoelectrode before and after removal of parylene from the tip. Parylene was found to be a stable insulating layer on the tungsten nanoelectrodes and these electrodes were used for all subsequent experiments.

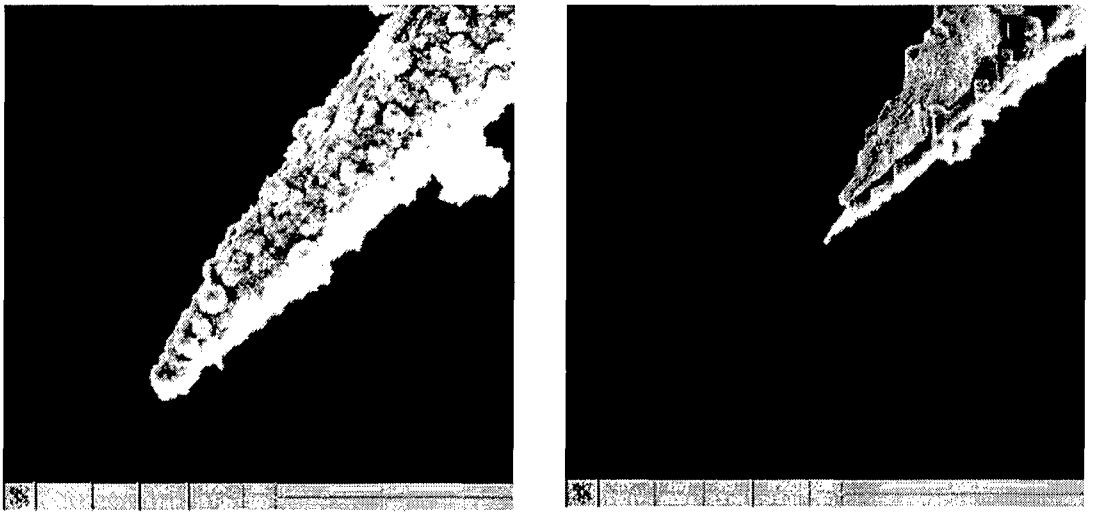


**Figure 4.14**: SEM Photographs of a parylene coated tungsten nanoelectrode before and after removal of parylene from the tip

## 4.2 Task 2: Equipment Modifications and Development

During this project, several sets of equipment were modified and developed to perform the various experiments. These setups are described in the following sections.

***4.2.1 Electron Beam Testing Setup:*** Figure 4.15 shows a photograph of an Electron Beam Tester that was setup at UHV during this project. This system was built by Lintech Corp, UK for testing silicon chips. Figure 4.16 shows a schematic diagram of various sub-systems and Table 4.1 lists the main specifications of this system. It is almost identical to a scanning electron microscope with some additional features. It contains a high voltage electron beam column for focusing the electrons emitted from a thermionic/field emission filament. It also contains beam deflection electronics to scan the beam over the sample. In addition, a high speed beam blanker is added to steer the beam into a 'dump' when it is not need. The beam blanker is critical to providing a pre-determined charge to the sample by making the beam of certain current hit a pad for a certain amount of time. This beam blanker is also used to create high speed e-beam pulses for electrical measurements. A high

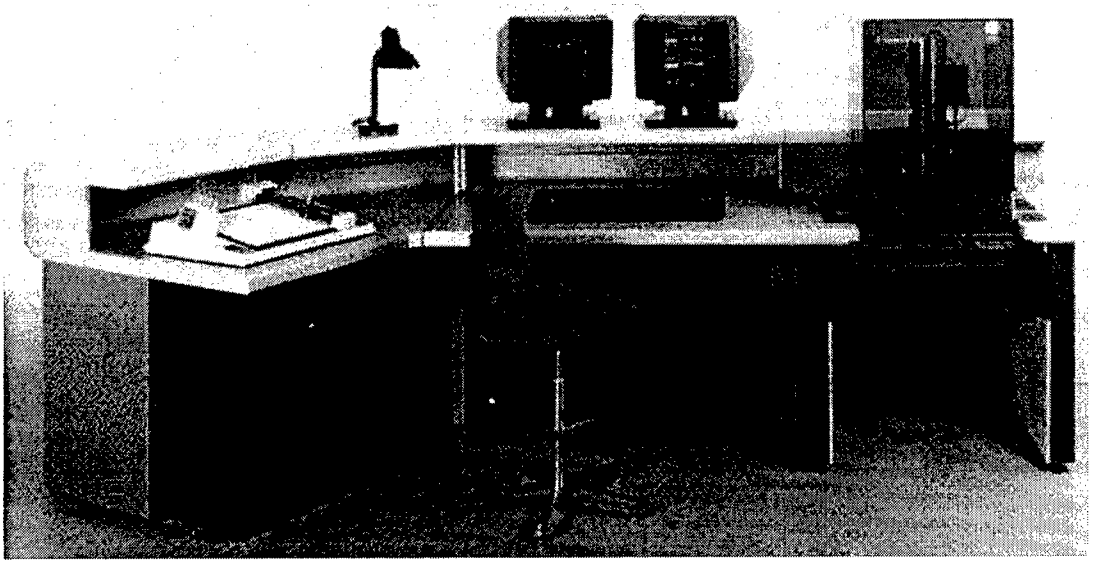


**Figure 4.15**: A photograph of Lintech electron beam tester for semiconductor chip testing that was setup at UHV during this project

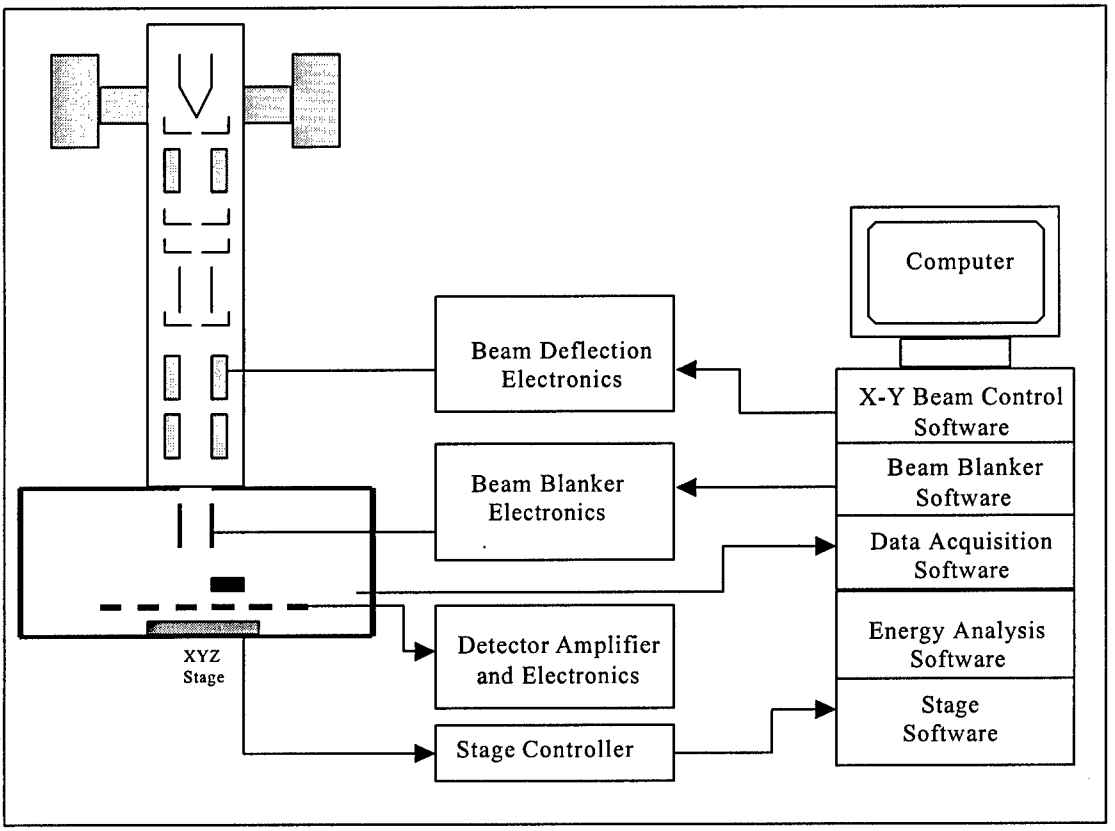



**Figure 4.16**: A block diagram of the e-beam addressing setup. Column drawing is not to scale.

**Table 4.1**: Specifications of Lintech EBT system at UHV

1. **Time Resolution** = 10% to 90% system rise time for step function input is 300pS (200pS FWHM sampling E-beam pulse)
2. **Voltage Resolution Limit** *in Waveform Mode* = 1 to 10 millivolts (specimen dependent)
3. **Voltage Resolution Limit** *in Imaging Mode* = 100 to 200 millivolts. The limit is set by the presence of contrast caused by topography and atomic number variation, as well as by signal to noise considerations.
4. **Low Beam Energy Spatial Resolution** (Imaging magnification depends on the working distance, but ranges from 30 X [covering a 10mm square DUT area] to 100,000 X). = 20 nm at 1 keV beam energy in real time imaging , 100nm in stroboscopic imaging at 700 eV beam energy
5. **Timing Comparison Resolution** = <100pS
6. **Linearity** in Closed Loop Voltage Measurement Mode = <1%
7. **Quantitative Voltage Measurement accuracy** in High Accuracy Mode = <5%
8. **Fine Digital E-beam Probe Positioning Resolution** = 20nm in a 1mm field
9. **Electron Gun Specifications**
   - ?? getter ion pumped directly heated Lanthanum Hexaboride (LaB6) cathode standard , automatic brightness optimisation and beam alignment , four electrode gun with constant extraction field
   - ?? brightness optimized in the range 300 eV to 3 keV (5 keV MAX)
   - ?? beam voltage can be set in 10 volt increments
   - ?? emission current variable in 5 microAmp steps
   - ?? three lenses with integral conjugate beam blanking double deflection scanning eight-pole electromagnetic stigmator , digital fine beam shift
10. **E-beam Pulsing**
    - ?? E-beam pulse widths (FHWM) of 200 picoseconds in 1:2:5 ratio to 10 microseconds
    - ?? trigger rates from 1 kHz to 250 mHz
    - ?? trigger impedance: 50 ohm high impedance
    - ?? time base from 5ns/division in 1:2:5 ratios to 20 microseconds/division; multipliers X1, X2, X5
    - ?? delay from trigger
    - ?? manual
    - ?? one shot
    - ?? swept, with a sweep time of 20 milliseconds in 1:10 ratios to 200 seconds
11. **Vacuum Chamber**
    - ?? Turbo- pumped Chamber 440mm wide X 440mm long X 330mm deep. This chamber can accommodate the integrated circuit under test installed on a large printed circuit board in order to exercise the DUT in its home environment. The circuit board is connected to the chip tester via the EBT air to vacuum connectors.
    - ?? X-Y stage : X +/- 80mm, resolution 5 microns / Y +/- 80mm, resolution 5 microns

resolution secondary electron detector with its associated electronics is used to image the sample in the scanning mode. In addition, an energy analyzer is used for generating the voltage contrast image and for determining the voltage on a pad. The system also uses a x-y-z stage typical of an SEM to accommodate large samples.

To use the NanoBench chips described in section 4.1.1 inside this electron beam tester, we designed and fabricated a vacuum sealed adapter as shown in Figure 4.17.

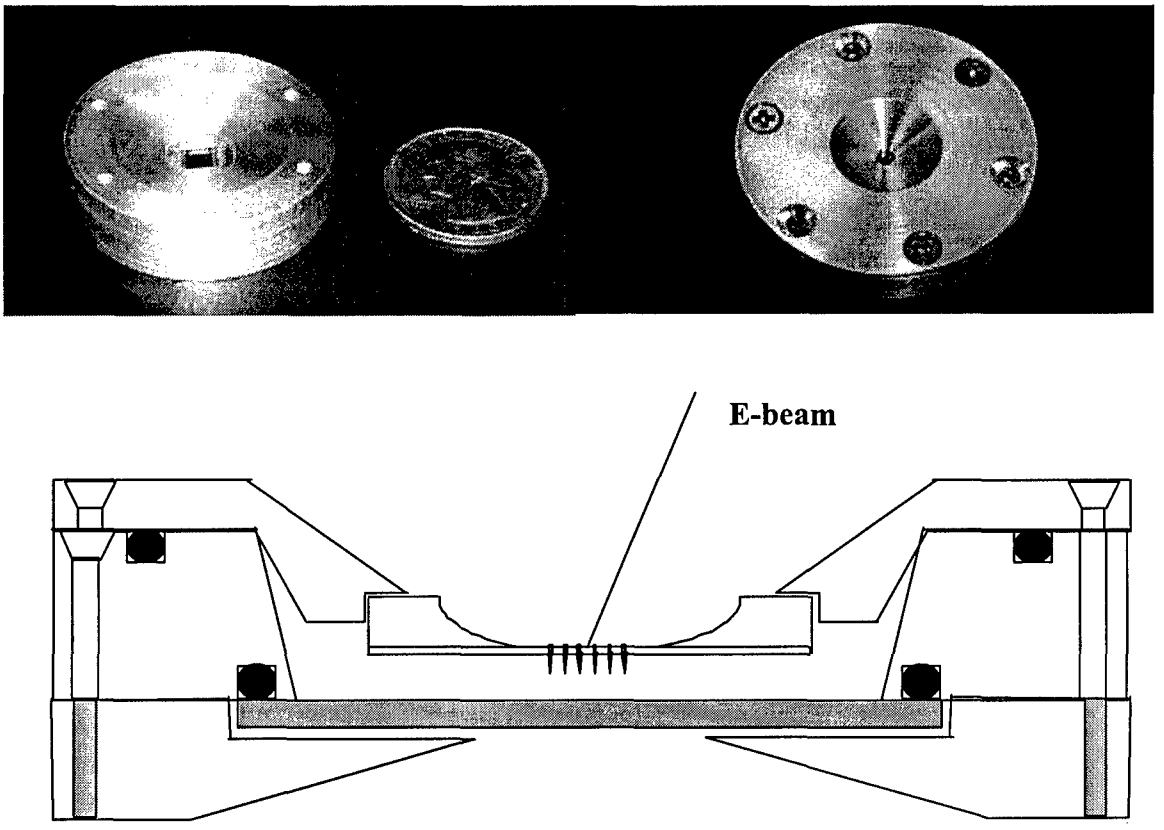



**Figure 4.17:** Photographs and a schematic diagram of the vacuum sealed holder for the 'through-hole' NanoBench chips

This holder is vacuum sealed with silicone o-rings in such a way that the cells can be kept alive in the medium while the e-beam hits the other side (vacuum side) of the NB chip. The overall size of this holder is only 1.5 inch diameter and thickness is 3/8 inch, thus it can be placed in almost any scanning electron microscope and useful data can be gathered with certain modification to the SEM. Figure 4.18 shows a schematic diagram how this holder will be used in a scanning electron microscope.

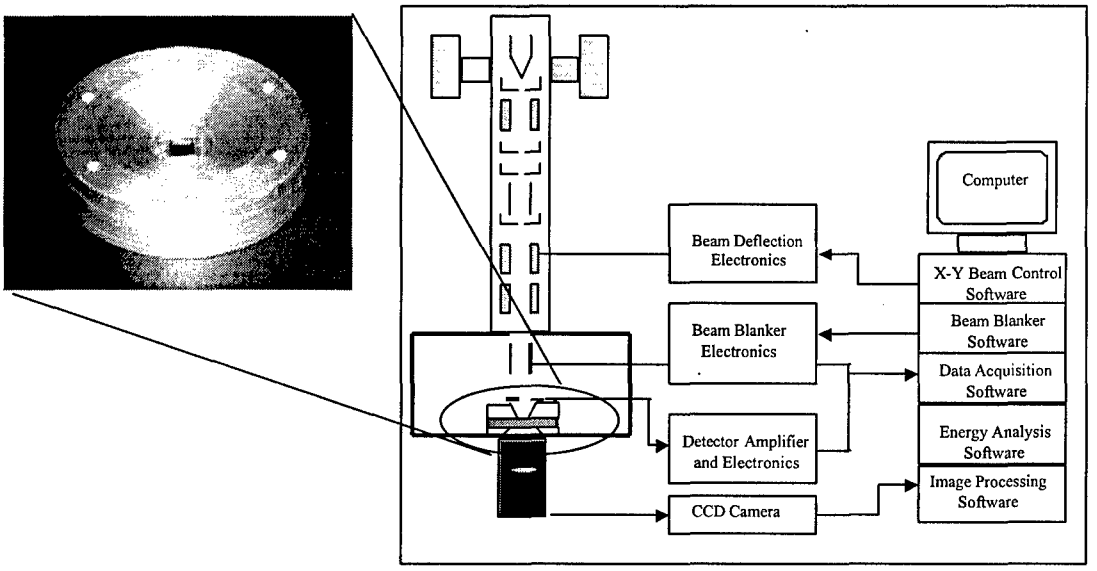



**Figure 4.18:** A schematic diagram of the setup for using the NanoBench holder developed during this project in a scanning electron microscope.

***4.2.2 Cell Probing Optical Microscope Setups:*** Two separate cell probing setups were assembled using Nikon microscopes. One of them was used for probing of cells using NanoBench chips described earlier while the other setup was used for probing cells with tungsten needles mounted on a Zyvex L100 nanomanipulator. Figures 4.19 and 4.20 show views of the first cell probing setup showing the NB chip mounted on a custom PCB held on the inverted microscope.

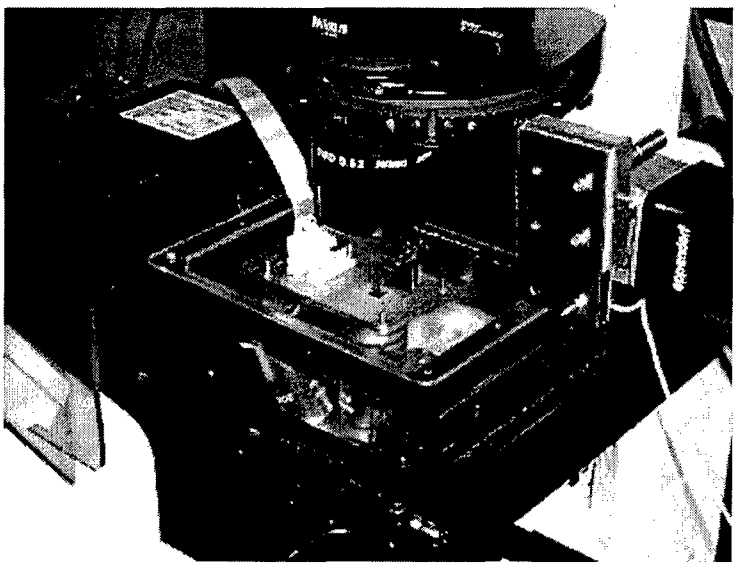


**Figure 4.19:** A photograph of the setup for probing cells with planar NanoBench chips

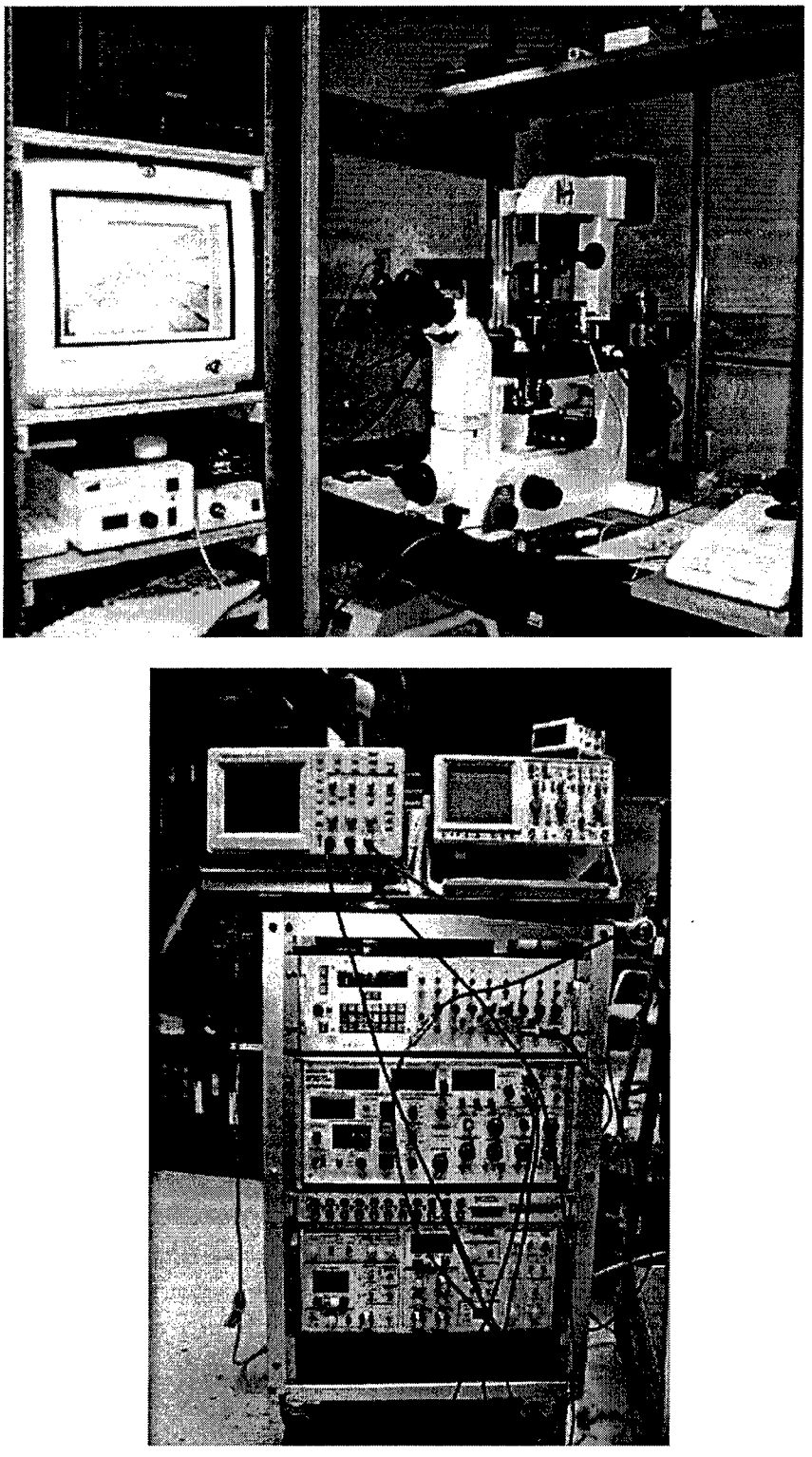


**Figure 4.20:** Photographs of optical microscope and electronic instrumentation used for recording signals when probing NRK cells and acute brain slices with NanoBench chips.

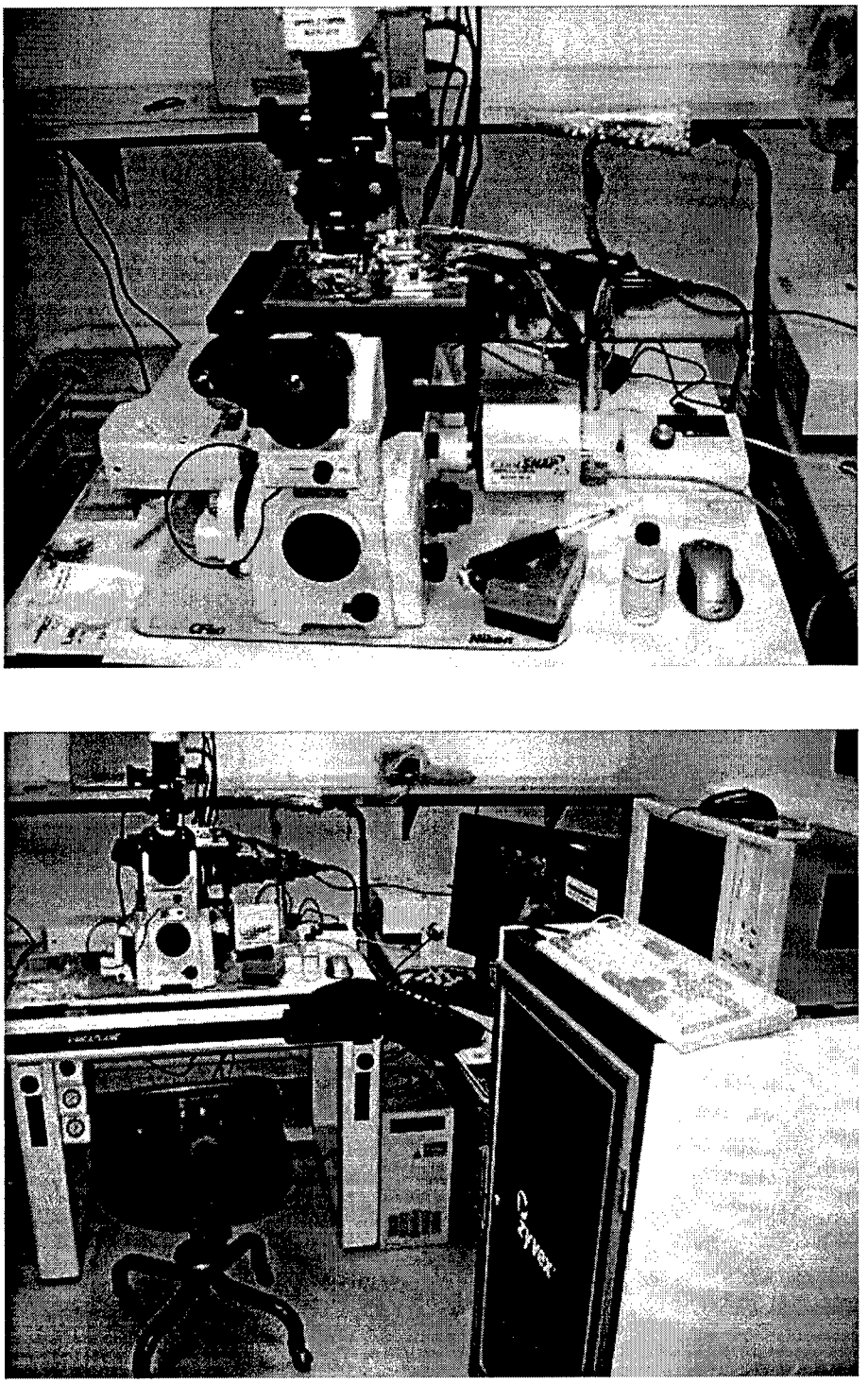


**Figure 4.21:** Photographs of Nikon TE2000 optical fluorescence microscope and Zyvex L100 nanomanipulator instrumentation used for probing cells with tungsten electrodes.

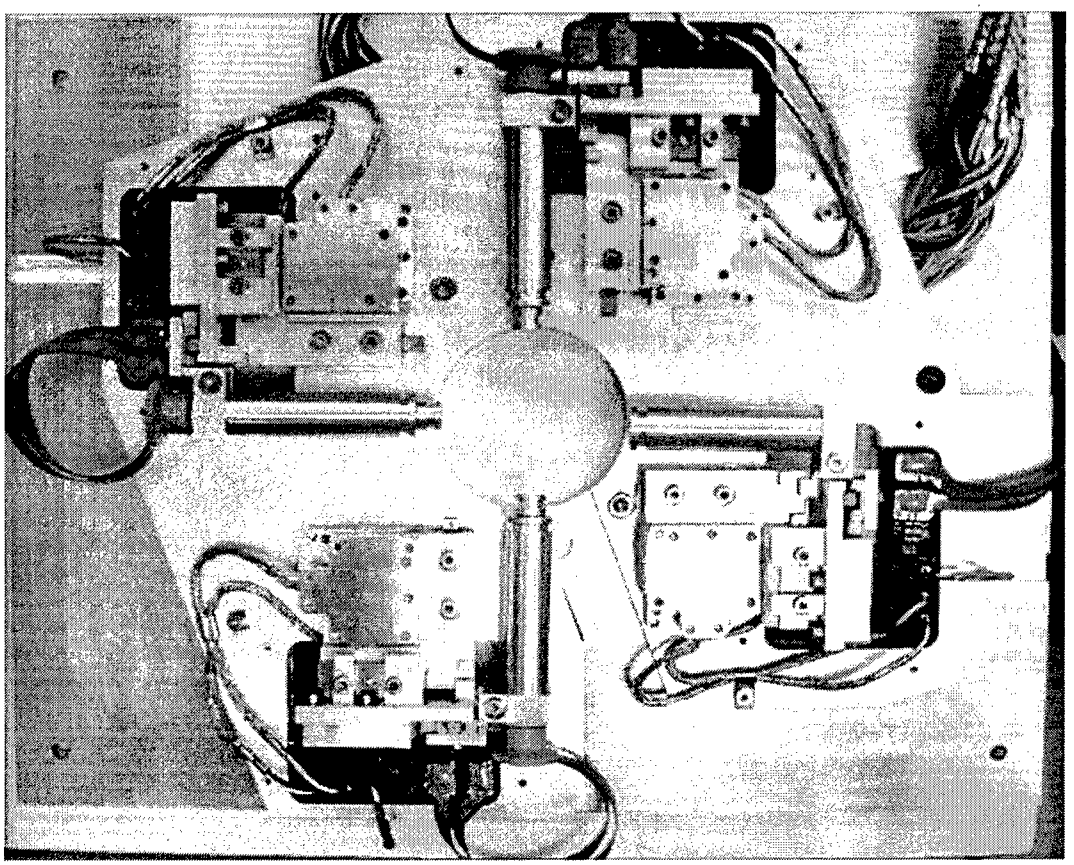


**Figure 4.22:** Photograph showing the details of four probe Zyvex L100 nanomanipulator used for probing cells with tungsten electrodes with sub-cellular resolution during this project.

Figures 4.21 and 4.22 show the second optical microscope consisting of a state-of-the-art Nikon TE2000 fluorescence inverted microscope and a Zyvex L100 nanomanipulator that was setup at UTD during this project. This setup was purchased through a grant from J. von Ehr foundation and we worked very closely with Zyvex to install and operate this system. The microscope is capable of both wide field and confocal fluorescence imaging to enable single molecule imaging. The nanomanipulator has a fine resolution of 5nm and can accommodate up to four tungsten needle probes. This system was extensively used during this project to perform various experiments that showed that it is possible to perform sub-cellular manipulations inside a cell. These experiments are described in later sections.

***4.2.3 Carbon Nanotube Probe Fabrication Setups:*** Figure 4.23-4.25 show photographs of a hot filament chemical vapor deposition (HFCVD) system for fabrication of multi-wall CNTs at UHV. The UHV baseline process for fabricating CNTs is described below.

This system was originally used for diamond thin film cathode development and was modified for CNT cathode fabrication. The system is essentially a plasma enhanced hot filament chemical vapor deposition (PE-HFCVD) system that uses a combination of methane, hydrogen and nitrogen to deposit CNTs. The system contains all the necessary gas handling cabinets and mass flow controllers for obtaining various gas composition necessary for process development. In addition, the substrate can be heated to temperatures as high as 900 degrees centigrade. The chamber is pumped with a large mechanical pump and the pressure can be independently (from the gas flow rate) controlled with a down-

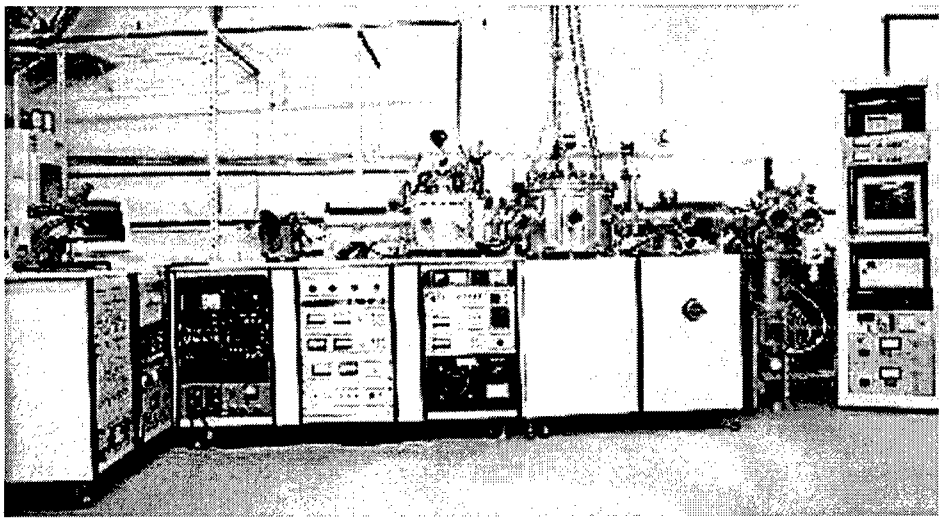


**Figure 4.23**: A photograph of the vacuum deposition system used for fabricating carbon nanotubes

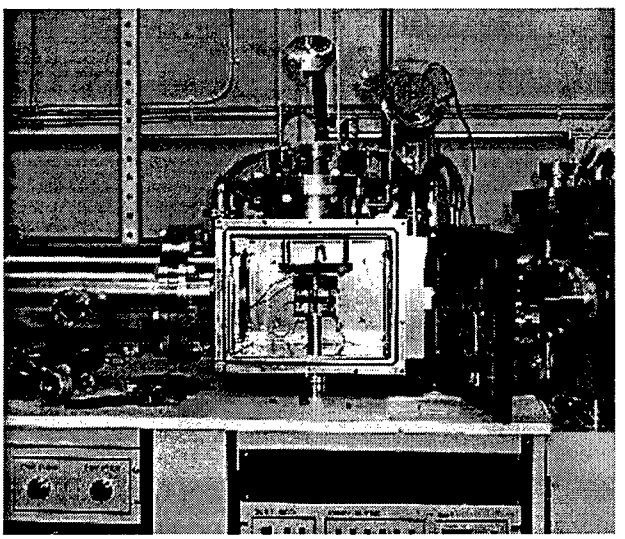


**Figure 4.24**: A photograph of the vacuum deposition chamber used for fabricating CNTs

stream throttle valve. The pressure is measured by a series of convectron and baratron gages. A series of hot tungsten filaments heated to temperature higher than 2000 degrees celcius is used to dissociate the process gases resulting in CNT growth. The PEHFCVD process involves passing a mixture of methane and nitrogen/hydrogen over white hot tungsten filaments. It is believed that the hydrogen turns into atomic hydrogen and creates the carbon containing radicals that cause nanotubes to be formed at the substrate surface. A DC plasma created between a Ta mesh (grid) and the substrate aids the nucleation and alignment of the nanotubes during growth. Using this method, aligned nanotubes can be deposited on heated substrates containing catalyst particles with the aid of the electrical field generated by the DC plasma. This method is also capable of producing aligned MWNTs at low substrate temperatures on catalyst pre-patterned substrates.

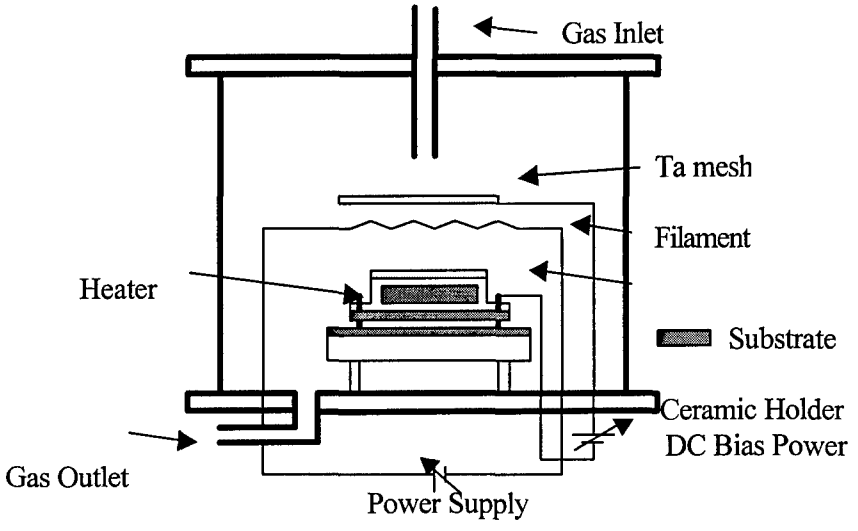



**Figure 4.25**: A schematic diagram of the PEHFCVD deposition chamber.

Figure 4.25 shows the schematic diagram of the PE-HFCVD chamber used for deposition of CNTs. Distinct from a conventional apparatus, a tantalum (Ta) mesh was installed above the filament at a distance of 15-20 mm, and a Mo wafer of 25 x 25 $mm^2$ was used as the substrate holder. A dc bias (both negative and positive) could be applied between the mesh and substrate holder by a dc power supply. A gas mixture of high purity nitrogen (99.999%) and methane (99.99%) was used as a gas source. The flow-rates were controlled using MKS mass flow controllers.

After the substrates (polycrystalline Ni wafer or stainless steel, usually, 2 pieces with the size of 5? 10$mm^2$ were used at the same time) were placed on the substrate holder, the vacuum chamber was pumped to 5 ? $10^{-6}$ Torr. The reactive gases ($N_2$ + $CH_4$) with the desired $CH_4$ concentration were flowed into the chamber to reach a pressure of 1-20 Torr. A throttle/needle valve was used to control the pumping speed and stabilize the pressure in

the chamber. Then, the filaments were heated to a desired temperature (1600–2200 ?C) and the DC plasma was generated. The overall process used (originally developed under a Phase I SBIR contract # DTRA01-00-P-0099) can be categorized in the following three steps:

1. Substrate Preparation: The Ni substrates used for the nanotube growth were polished by # 4000 SiC sanding paper before putting them into the vacuum chamber. The careful treatment is important for the generation of the uniform catalyst particles. If the surface is not treated well, the diameter of the nanotubes is not uniform. It should be mentioned here that the diameter of the nanotubes is controlled by the size of the catalyst particles.

2. Plasma Treatment: DC plasma generated on the surface of the substrates was used to form the controllable small catalyst particles, which led the aligned growth of carbon nanotubes. For the catalyst containing substrate such as the Ni wafers and Ni coated Si, a strong plasma (600V) was used to bombard the surface for less than 10 min to generate the particles. This process makes the deposition of nanotubes very simple, and avoids the tedious catalyst particle preparation used by other research groups who used a lengthy procedure for preparation of the meso-particles on a porous silicon surface. DC plasma is the key factor that affects the growth of nanotubes. It is the DC plasma that makes the hot filament CVD (HFCVD) very successful in the synthesis of the nanotubes. The main effects of the DC plasma can be described as:

(i) Possible mechanism for the aligned growth: The mechanism of the DC plasma based aligned growth of nanotubes has been described in the previous subsection. The DC plasma current is essential for the plasma to work. If no plasma is generated, i.e., only voltage applied between the cathode and anode with no plasma generated, there is no growth of the aligned nanotubes. This is one of the reasons that some of the initial experiments failed.

(ii) Generation of the small catalyst particles: We have established a technique to produce catalyst particles of correct size directly on the substrate surface by using a high plasma intensity process. A mixture of $N_2$ + $CH_4$ ($CH_4$ concentration is in the range of 3-10 vol%) was used as the reactive gas source. During this nucleation stage, strong plasma was generated (by applying the highest voltage the DC power supply can provide) for 8 min. When the plasma intensity is not high enough, the nucleation rate will be very low. When 400V?0.05A was applied during the nucleation stage, the nucleation (generation of the catalyst particles) took 30-50 min. The $CH_4$ in the plasma is also very important for the generation of catalyst particles. If only a $N_2$ plasma was used, there were no catalyst particles formed. We believe that the carbon diffusion into the catalyst particles helps the plasma to pull the particles out from the substrate surface.

3. Nanotube deposition: After the plasma treatment to produce desired nucleation sites was finished, a nanotube deposition process was used to grow the aligned nanotubes. These nanotubes grew perpendicular to the substrate surface. A mixture of nitrogen and CH4 in presence of DC plasma and heated filament was used to achieve the nanotube growth. During this project, we started with the baseline process developed at UHV. After

establishing the base line process, we studied the effect of pressure, nanotube deposition time, filament temperature and various substrates on the nanotube growth. Figure 4.26 shows several SEM photographs of CNTs grown at UHV.

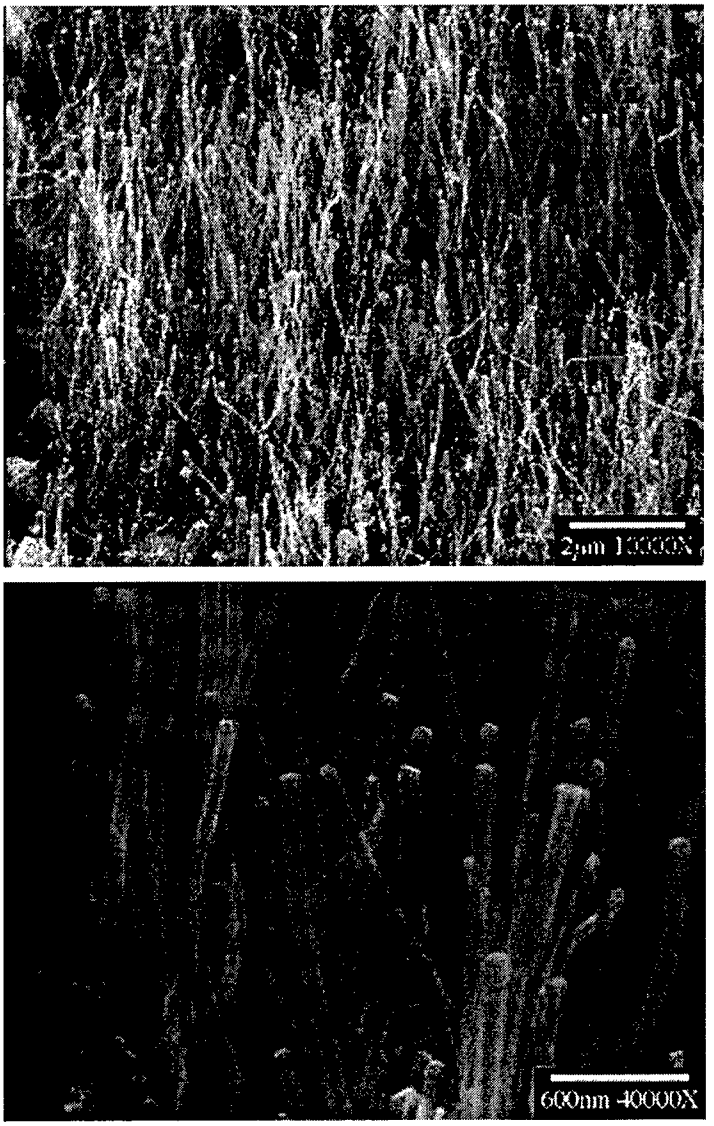



**Figure 4.26:** Several SEM photographs of CNTs grown at UHV.

## 4.3 Task 3: Testing of NanoBench Chips and Nanoelectrodes

The NanoBench chips and nanoelectrodes fabricated during task 1 were tested and evaluated at various stages of fabrication process as discussed earlier in section 4.1. However, the main electrical performance was tested after complete fabrication. The following parameters were measured.

1. Leakage current
2. CNT charging
3. Voltage reading
4. Impedance measurement
5. Waveform testing
6. Signal to noise

**Results from a NanoBench Chip:**

| | |
|---|---|
| Number of electrodes: | 16 |
| Electrode size: | 5 um x 1.5um |
| Insulator: | Parylene |
| Insulator thickness: | 1 um |
| Frequency: | 1 kHz |
| Electrode to ground impedance: | 15 M-ohms (in medium) |
| Electrode to electrode impedance: | >30 M-ohms |

This is an acceptable value for measuring cell membrane potential.

**Results from Tungsten needles with insulating shank:**

| | |
|---|---|
| Number of electrodes: | 1 |
| Electrode tip size: | ~100nm |
| Length of removed sheath: | 5 um |
| Insulator: | Parylene |
| Insulator thickness: | 1 um |
| Frequency: | 1 kHz |
| Electrode to ground impedance: | >50 M-ohms (in medium) |

This impedance value is similar to those obtained from glass Ag-AgCl electrodes, typically used for electrophysiological measurements.

## 4.4 Task 4-6: Functionalization of Nanoelectrodes for Sub-cellular studies

Most of the time and effort during this short project was spent on setting up the experimental setups since they did not exist at all. However, we were able to able to functionalize tungsten probes and use them to demonstrate sub-cellular studies.

***4.4.1 Nanoelectrode Insertion in Cells***: As described earlier, probe preparation is crucial for cell insertion applications and consists of electrochemical etching of tungsten. This step involves controlling the electrical potential and withdrawal rate from the etching solution to make the most desirable shaped probes, which are usually slender with very gentle tapers to a sharp tip. The gentle taper is crucial to avoid hitting the broad part of the probe on the dish before the tip can be steered to the target cell site. We found that if the tip is smaller than 100nm, it penetrates into the cell without causing damage to the cell. On the other hand, if the tip size is larger than 150nm, the cell gets easily damaged. The Zyvex manipulator allows insertion of four probes at a time as shown in Figure 4.27 below.

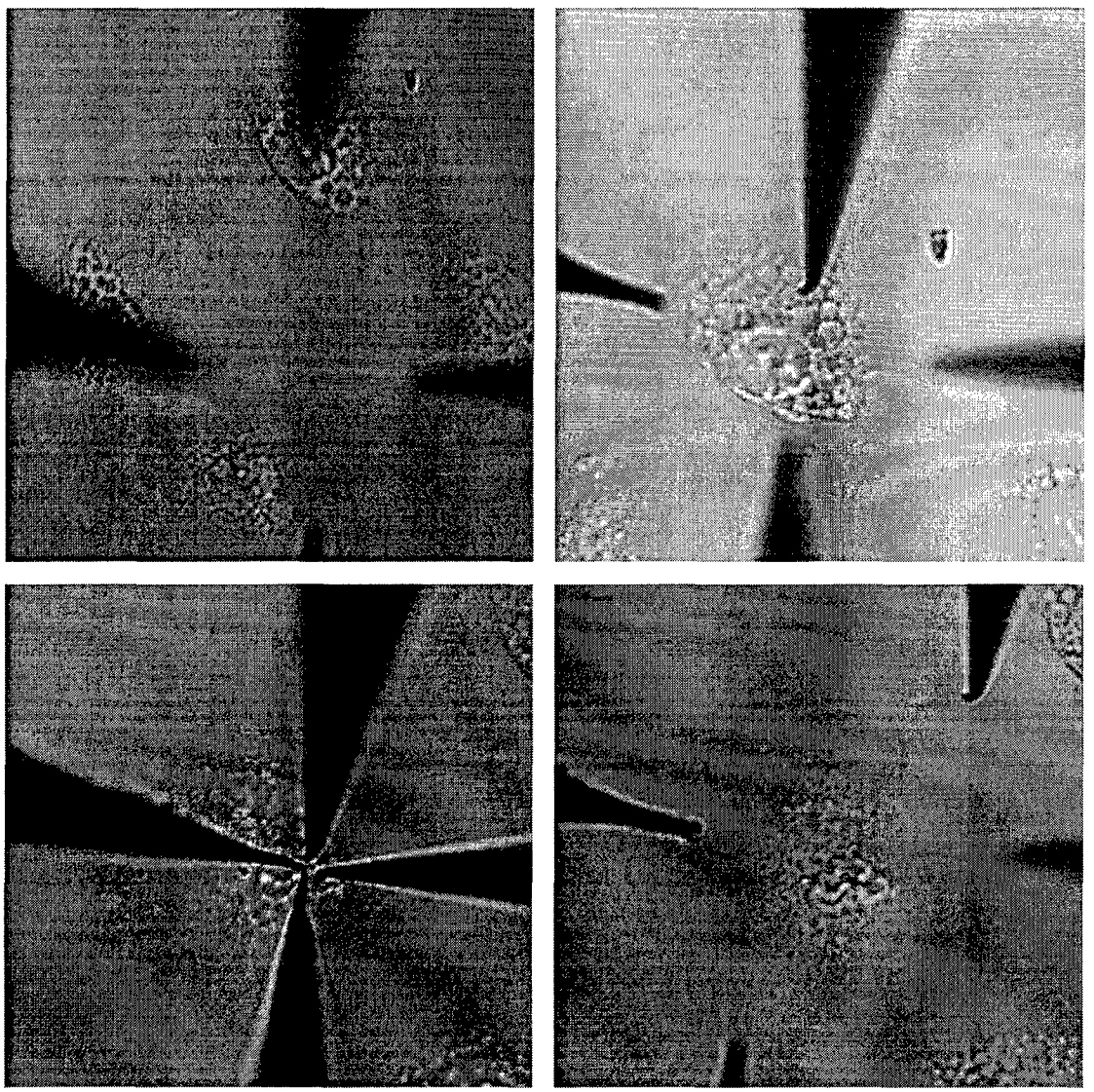


**Figure 4.27:** Optical microscope photographs of a cell being probed with four independently controlled metal probes on the TE2000-L100 experimental setup. (a) Before touching, (b) probes closer to the cell, (c) probes touching the cell, and (d) probes moved up after touching the cell showing damaged cell.

In this particular experiment with four probes, the probe tips were pretty large and thus caused damage to the cell. Figure 4.28 shows a reconstructed picture of a very fine probe inserted into a NRK cell.

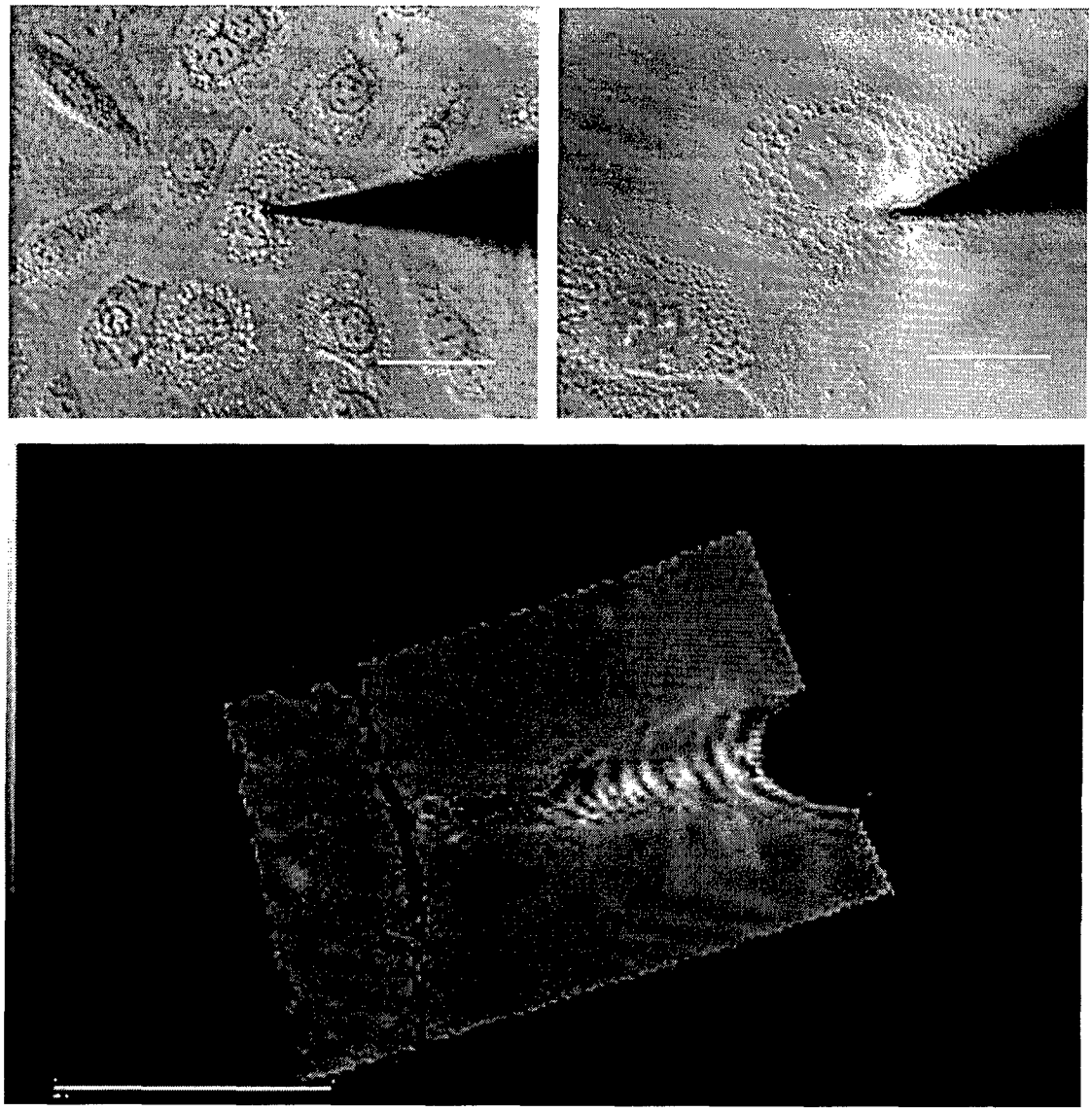


**Figure 4.28:** Optical microscope photographs and a computer reconstructed picture of a cell being probed with a very fine metal nanoelectrode, clearly showing the needle penetrating the cell wall.

***4.4.2 Nanoelectrode Functionalization***: After tungsten nanoelectrode sharpening, the second step, when needed, is to plate the tungsten probes with gold by evaporation. As mentioned earlier, a focused ion beam was also used to sharpen probes and then coat them with platinum by metal deposition in the same system. Figure 4.29 shows SEM micrographs of a tungsten probe before and after platinum coating inside the FEI focused ion beam system.

The next step is to functionalize the gold (or platinum) coated probes with biological material, such as antibodies, DNA, or other fluorescent material. Figure 4.30 shows a gold-coated probe on which a monolayer of thiol-terminated alkane containing biotin was reacted with fluourescent streptavidin. This demonstrates the production of a fluorescent probe.

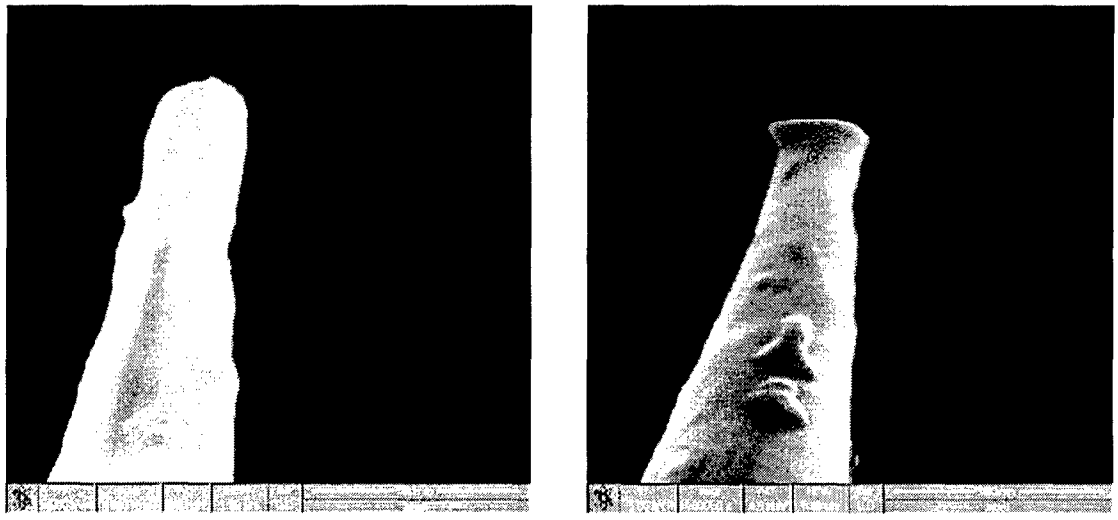


**Figure 4.29:** SEM micrographs of a tungsten probe before and after platinum coating inside the FEI focused ion beam system.

**Figure 4.30:** Photographs of a tungsten probe on which a monolayer of thiol-terminated alkane containing biotin was reacted with fluourescent streptavidin (a) DIC image and (b) fluorescence image (scale bar is 25 um).

***4.4.3 Intra-Cellular Experiments with Nanoelectrodes***: To demonstrate the feasibility of manipulating intra-cellular structure with these probes, we prepared sucrosomes in NRK cells by incubating the cells with 30 mM sucrose to swell lysosomes. Figure 4.31 (a) below shows a probe positioned to penetrate a sucrosome (marked with the arrow). Figures 4.31 (b)-(d) show the slow insertion of the probe into the sucrosome. Figures 4.31 (e)-(i) show the withdrawal of the probe from the sucrosome. Note that the sucrosome stays intact until the probe is withdrawn, then collapses to a smaller structure (marked with the arrow in Figure 4.31 (i)).

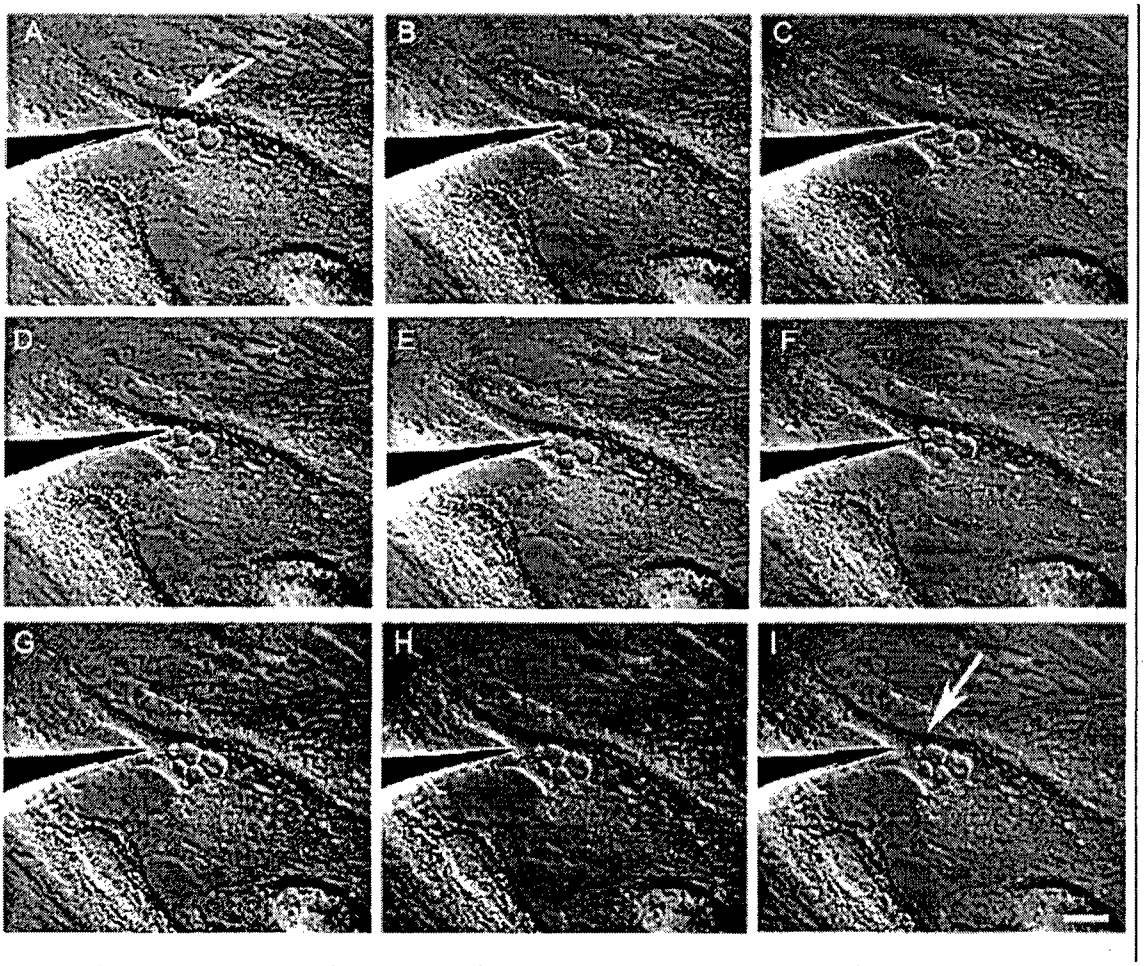



**Figure 4.31:** Sequential photographs of a tungsten nanoelectrode being inserted and removed from a NRK cell, showing the capability of instruments developed during this project.

In another experiment, lysosomes in NRK cells were visualized with the fluorescent dye LysoTracker Red (Invitrogen/Molecular Probes). A probe was inserted into cells and slowly maneuvered towards a fluorescent vesicle. The probe itself is unlabeled and seen only as the faint shadow marked by the arrowhead in panels 3-7. In panel 8, the probe tip hits the lysosome, which bursts, shown by the loss of the fluorescence in panels 8 and 9.

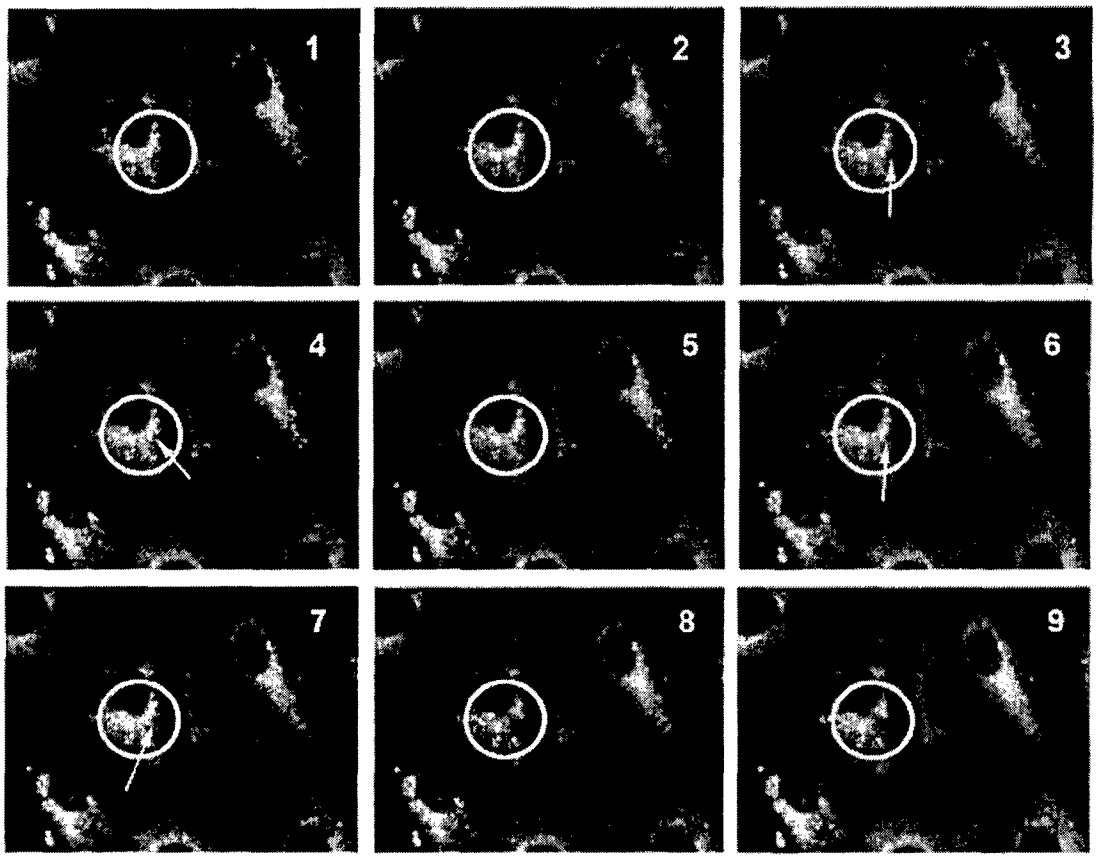



**Figure 4.32:** Sequential photographs of a tungsten nanoelectrode being inserted into fluorescent vesicles.

## 4.5 Conclusions

In conclusion, an innovative approach called NanoBench was investigated during this project. NanoBench chip consists of an array of electrically conducting nanoelectrodes that can be inserted inside a cell to measure intra-cellular signaling pathways, both electrically and optically. During this project, (i) NanoBench chips with arrays of metallic tips were fabricated, (ii) very sharp tungsten probes were fabricated, functionalized and used to demonstrate intra-cellular probing of cells, and (iii) full range of experimental techniques have been developed to perform optical and electrical measurements of intracellular signals.

As a model system, a sharp tungsten probe was inserted into a lysosomes that was swollen with sucrose (a sucrosome). Control over probe movement was sufficient to drive the probe into a sucrosome, and withdraw the probe. Upon withdrawal, the sucrosome collapsed, indicating that the vesicle was punctured by the probe tip. In a second model system, a lysosome labeled with the acidotropic dye LysoTracker Red was touched with a metal tip, which popped the lysosome and released the dye. Future work will concentrate on making sharper probes and coating them with biological molecules to sense analytes in cells.

## 5.0 Personnel Supported

During this project, the following personnel worked on this project:

**UHV Technologies, Inc.**

Nalin Kumar, Ph.D., Principal Investigator
Ali Ouali, fabrication of CNTs
Sam Duffy, design and fabrication of various mechanical fixtures.
Jitender Singh, fabrication of mechanical fixtures and experimentation
Octavio Herrera, equipment assembly and testing.
Manual Garcia, fabrication of tungsten nanoelectrodes.

**Genome Data Systems, Inc.**

Rajan Kumar, MD, Ph.D., Functionalization and testing of nanoelectrodes

**University of Texas at Dallas**

Prof. Bruce Gnade, fabrication of NanoBench
Prof. Moon Kim, fabrication of NanoBench
Andrew Gnade, Student, fabrication of NanoBench
Tom Brock, Summer intern, fabrication of NanoBench
Prof. Rockford Draper, Functionalization of nanoelectrodes
Dr. Carole Mikoryak, Preparation of NRK cells
Prof. L. T. Thompson, Electrophysiological measurements

## 6.0 Publications

None.

## 7.0 Interactions/Transitions

None.

## 8.0 New Discoveries, Inventions and Disclosures

1. A provisional patent application entitled ' Advanced Microelectrodes and Nano-electrode Arrays' was filed on 4/16/04.

2. A non-provisional patent application entitled 'Individually Addressable Nanoelectrode Array' was filed on 7/14/04. This application has a priority date of 7/15/03.

## 9.0 Honors/Awards

None.